2003

Micropropagation of Carnivorous Plants

Kenneth Stephen Uhnak
University of Rhode Island

MICROPROPAGATION OF CARNIVOROUS PLANTS

BY

KENNETH STEPHEN UHNAK

A DISSERTATION IN PARTIAL FULFILLMENT OF THE

REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOLOGICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2003

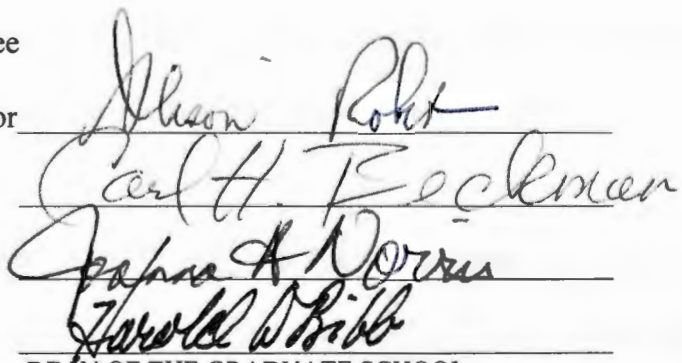
DOCTOR OF PHILOSOPHY DISSERTATION

OF

KENNETH S. UHNAK

APPROVED:

Dissertation Committee

Major Professor 

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2003

ABSTRACT

A simple, efficient system for seed surface disinfestation and *in vitro* germination was developed for the carnivorous pitcher plants *Darlingtonia californica* and *Sarracenia leucophylla*. Of the disinfectants tested, hydrogen peroxide or 10% Clorox® were most effective for disinfesting seeds of *D. californica*, while concentrated sulfuric acid worked best for *S. leucophylla.* Differences in the effectiveness of sterilants were associated by differences in seed coat morphology. Seeds of *D. californica* imbibed at 4-7°C in sterile deionized water with surfactant and gibberellin germinated earlier than seeds without exposure to gibberellin. Unimbibed seeds of *S. leucophylla* germinated rapidly in sterile water after treatment in concentrated sulfuric acid.

Scanning electron microscopy of *D. californica* seed coats revealed waxy trichomes covering the seed surface. In contrast, the seed coats of *S. leucophylla* were pitted with surface and sub-surface cells possessing heavily thickened cell walls. These cells were devoid of contents. Fungal hyphae were observed on the seed surface and within empty cells of the integuments. Scanning electron microscopy observations and comparison of seed coat morphology of the carnivorous plant genera *Drosera, Dionaea, Sarracenia* and *Darlingtonia* revealed a wide range of differences in structure and ornamentation, which may suggest a species specific approach to surface disinfestation.

A simple effective system for *the in vitro* growth, multiplication and rooting of axenically germinated seedlings of *D. californica* was developed. Seedlings grown on solid ½ strength Murashige and Skoog medium produced more biomass and more and

longer pitcher leaves than seedlings grown on other solid media assayed. Root development on solid media was minimal and usually limited to the seminal root regardless of the medium. Seeds stimulated by gibberellic acid prior to germination and exposed to auxin and cytokinin during early seedling development produced multiple offshoots as well as fibrous root systems when transferred to ½ strength liquid medium containing charcoal. Similarly treated seedlings transferred to ½ strength liquid media without charcoal produced multiple offshoots but fewer root systems. Seedlings cultured in medium without charcoal produced more but smaller pitchers than seedlings cultured in medium containing charcoal. Multiplication did not occur on solid media, and seedling growth was stunted. Seedling multiplication through offshoots occurred in all liquid media and was both prolific and rapid. *Darlingtonia californica* was regenerated from whole, *in vitro* germinated seedlings and excised segments from *in vitro* generated juvenile pitchers. When incubated on solid Phytomax Orchid Multiplication Medium, seedlings produced protocorm-like bodies and green leafy callus When divided and subcultured in liquid Phytomax Orchid Multiplication Medium, explants of both protocorm-like bodies and green, leafy callus gave rise to multiple shoots as well as more protocorm-like bodies and green, leafy callus. These could be further divided and subcultured. Transverse segments of excised pitcher leaves from axenically-grown seedlings produced shoots and protocorm-like bodies when subcultured in liquid Phytomax Orchid Multiplication Medium. Unlike *D. californica*, seedlings of *S. leucophylla* did not readily produce offshoots when incubated on solid media. A protocol for extraction of embryos from selected *Sarracenia* species was developed.

ACKNOWLEDGEMENTS

I especially thank my major professor Dr. Alison Roberts for her guidance, patience and vision in helping me bring this project to fruition. I thank her for believing in me, and for keeping me focused during difficult times (of which there were many). This must not have been easy for her.

I thank the members of my committee, Dr. Richard Koske for his guidance and advice throughout the years, Dr. Joanna Norris for her exceptional patience, and Dr. Carl Beckman whose spirituality has left its mark on me. I also thank Dr. Larry Englander for acting as chair of my defense.

I thank Chris Nerone, Veronica Masson, Marybeth Able, Stacie Paquette, Jane Leary, my sons Kenneth and Nicklaus Uhnak, John Phillip Jr., and Judy Gould. I thank Ranger Hall graduate students Ryan Tainsh and Mike Budziszek for helping with statistics and computer problems, Alissa Neill for support, and Mary Beth Hanley for kindly leaving food in the refrigerator on nights I worked until dawn. I thank Dr. Eric Roberts for generously saving me from certain destruction during the final hours.

I thank the Alley family for believing in me enough to offer their prayers of support. I thank William Alley, Janice Alley, and William Alley Jr. I also thank Claudia, Becky, Ron and Nana Franke for their much appreciated support and prayers. I thank my parents Dominic and Bernadette for their love and support.

A special thank-you is due to my wife, Leslie Anne Uhnak, who not only typed much of this dissertation (more than once), but also endured a somewhat chaotic life during its completion. I also thank her and my stepdaughter, Kelsey Desrosiers, for their support and belief in me during this remarkable ordeal.

PREFACE

This dissertation was written in the manuscript style format as approved by the Graduate School of the University of Rhode Island with modifications as required for submission to the journal. *Plant Cell Tissue and Organ Culture*. Three manuscripts are included.

Manuscript I:

ESTABLISHMENT OF A MICROPROPAGATION SYSTEM FOR MEMBERS OF THE CARNIVOROUS PLANT FAMILY SARRACENIACEAE. I. SURFACE STERILIZATION AND *IN VITRO* GERMINATION OF SEEDS OF *DARLINGTONIA CALIFORNICA* WITH NOTES AND OBSERVATIONS ON *SARRACENIA LEUCOPHYLLA, S. ALATA, AND S. PURPUREA*

Manuscript II:

ESTABLISHMENT OF A MICROPROPAGATION SYSTEM FOR MEMBERS OF THE CARNIVOROUS PLANT FAMILY SARRACENIACEAE. II. *IN VITRO* GROWTH AND EARLY SEEDLING DEVELOPMENT OF THE NORTH AMERICAN PITCHER PLANT *DARLINGTONIA CALIFORNICA*

Manuscript III:

ESTABLISHMENT OF A MICROPROPAGATION SYSTEM FOR MEMBERS OF THE CARNIVOROUS PLANT FAMILY SARRACENIACEAE. III. MORPHOLOGICAL RESPONSES DURING *IN VITRO* REGENERATION OF THE NORTH AMERICAN PITCHER PLANTS *DARLINGTONIA CALIFORNICA* AND *SARRACENIA PURPUREA*

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Manuscript I

Page

Manuscript II

Manuscript III

LIST OF TABLES

Page

MANUSCRIPT I

ESTABLISHMENT OF A MICROPROPAGATION SYSTEM FOR MEMBERS OF THE SARRACENIACEAE

I.

DISINFESTATION AND *IN VITRO* GERMINATION OF SEEDS OF THE NORTH AMERICAN PITCHER PLANTS *DARLINGTONIA CALIFORNICA* TORREY, *SARRACENIA LEUCOPHYLLA* RAF., AND *S. PURPUREA* L.

ABSTRACT

A simple, efficient system for seed surface disinfestation and *in vitro* germination without lengthy seed stratification was developed for the carnivorous pitcher plants *Darlingtonia californica* and *Sarracenia leucophylla*. Of the disinfectants tested, hydrogen peroxide or 10% Clorox® were most effective for disinfesting seeds of *D. californica*, while concentrated sulfuric acid worked best for *S. leucophylla*. Differences in the effectiveness of sterilants were associated by differences in seed coat morphology. Seeds of *D. californica* imbibed at 4-7°C in sterile deionized water with surfactant and gibberellin germinated earlier than seeds without exposure to gibberellin. Unimbibed seeds of *S. leucophylla* germinated rapidly in sterile water after treatment in concentrated sulfuric acid. Scanning electron microscopy of *D. californica* seed coats revealed waxy trichomes covering the seed surface. In contrast, the seed coats of *S. leucophylla* were pitted with surface and sub-surface cells possessing heavily thickened cell walls. These cells were devoid of contents. Fungal hyphae were observed on the seed surface and within empty cells of

the integuments. Scanning electron microscopy observations and comparison of seed coat morphology ofmembers of the carnivorous plant genera *Drosera, Dionaea, Sarracenia* and *Darlingtonia* revealed differences in structure and ornamentation, which may suggest species-specific approaches to surface disinfestation.

INTRODUCTION

Since the time of Darwin the various genera of carnivorous plants have fascinated both botanists and plant enthusiasts (see Lloyd, 1942, for early references). Their many unique anatomical and physiological adaptations peculiar to the carnivorous habit continue to attract attention from workers in such diverse areas as developmental biology, ecology, cell biology, micropropagation and evolutionary biology.

This artificially grouped assemblage of plants comprises more than 600 species (Adamec, 1997) and has grown considerably since 1989 when Givnish reported 538 species (Table 1, Appendix B). Taxonomically, species of carnivorous plants occur in nine families and 19 genera (Table1, Appendix B). Micropropagation of members of this group is desirable for several reasons. Several members of the genus *Drosera* and the related monotypic genus *Dionaea* are a source of medicinal compounds (Bobak et al., 1995; Bobak et al., 1993; Budzianowski, 2000, 1996, 1995a, 1995b; Crouch et al., 1990; Hook, 2001; and Zenk and Steglish, 1969). Additionally, extracts of the roots of *Sarracenia flava* possess antitumor activity against certain forms of human cancer cells (Miles et al., 1974). Collection pressure and/or habitat destruction has reduced or depleted many natural populations of carnivorous plants in the wild (Anthony, 1992;

containing levels of nickel, zinc, chromium and other metals that are generally phytotoxic makes it extremely interesting and appropriate for the study of what may be unique developmental and physiological processes. *S. leucophylla* is an American pitcher plant found on the lower Gulf Coast of the United States in Georgia, Florida, Alabama and Mississippi.

Although several species of carnivorous plants have been micropropagated (see appendix C this dissertation), work on members of the Sarraceniaceae has been limited. A search of the literature revealed only two papers on their *in vitro* culture. Withner (1964) reported qualitative observations on applying the techniques of orchid culture to *D. californica* and *S. purpurea*. *D. californica* also was included in a broad survey of the feasibility of micropropagation of carnivorous plants (Boulay, 1995). Until recently few reports have addressed the germination of seeds of carnivorous plants, particularly members of the Sarraceniaceae. Prior to the publication of Ellison's (2001) extensive study on the germination of 8 species of *Sarracenia*, the literature contained only two papers (not including Withner's note) on the germination of seeds of only one specie, *S. purpurea* (Mandossian, 1966; Gotsch and Ellison, 1998). No micropropagation systems for the Sarraceniaceae have been described in the literature. This is surprising since many of the species that are offered commercially have been propagated by tissue culture. The lack of published information on propagation of these species likely reflects the concerns of commercial producers who consider such techniques proprietary (a brief search of the web on 4/12/03 found four commercial suppliers offering carnivorous plant species from tissue culture including members of the genera *Sarracenia, Heliamphora*, and

Nepenthes). I feel that development of such systems and publication of the protocols will help alleviate collection pressure in the wild and offer other workers in the field methods that may facilitate the study of developmental, physiological, and ecological questions whose answers may be found within the unique adaptations and habit of this marvelous group of plants.

This manuscript reports on the initial steps in the establishment of a micropropagation system for members of the carnivorous plant family Sarraceniaceae.

wash, seeds were resuspended in 2-3 ml of sterile deionized water and transferred to sterile glass petri dishes. Fine forceps or a sterile bacterial loop was used to transfer the seeds (one per well) to individual wells of 12 or 24-well sterile plastic culture plates (Sigma, St. Louis, Missouri) with each well containing 2 ml of sterile MYP (malt extract, 7.0 g/l; yeast extract, 0.5 g/l; bacto-peptone, 1.0 g/l; Koske, 1977) and 10 g/l sucrose. Medium pH was adjusted to 6.5 with KOH before the addition of 9.5 g/l Bacto-agar (Difco, Detroit, Michigan) and autoclaved at 121°C for 15 min. Forty-eight seeds were used in each treatment. Experiments were repeated twice using two different seed lots for each species. Seeds were purchased in 2001 and 2002 respectively. All seeds were collected by the supplier in the fall of each year and stored at 4-7°C prior to shipment (personal communication, Peter Paul's Nurseries, Canadaigua, NY). Culture plates were wrapped in Saran Wrap® brand plastic wrap (Dow Brands, Indianapolis, Indiana), sealed in a Rubbermaid® container (Newell Rubbermaid Inc., Freeport, Illinois) and placed in a dark growth chamber at a constant temperature of 27°C. Cultures were examined for signs of contamination with the aid of a dissecting microscope daily or every other day for a period of 15 days . Additionally, 48 seeds of *S. purpurea* and 24 seeds each of *S. flava*, *S. alata* and *S. rubra* were subjected to the sterile wash series in treatment #5 as described above.

Scanning Electron Microscopy

For surface studies, seeds of *Darlingtonia, Sarracenia, Drosera* and *Dionaea* spp. were air-dried for 24 h then sputter-coated with gold during rotation for 90-160 s. For internal studies, seeds were cut in half or into sections using a razor blade or fine pointed surgical scalpel and a dissecting microscope. Any residual endosperm or

embryonic tissue was removed using a fine pointed dental pick. Cut seeds were air-dried for 24 h, fixed to double sided tape (Scotch) on microscope coverslips, sputter coated with gold and attached to the stage mount with double sided tape. Seeds of *D. californica* and *S. leucophylla* also were treated for 4, 8, 12, and 16 min in concentrated H_2SO_4, rinsed 3 times in sterile deionized water and air-dried for 24 h before being prepared for SEM as described above. Observations were made with an Hitachi 4100 Scanning Electron Microscope (Hitachi, Ltd., Toyo, Japan). Images were captured using a Sunspark Image Capture System (Sun Microsystems Inc., Santa Clara, California).

Seed Germination Studies

Six methods used to germinate seeds are summarized in table #1. Germination was scored by emergence of the radicle.

Germination of Unimbibed Seeds in Liquid Culture

Seeds of *D. californica* and *S. leucophylla* were surface-disinfested for 10 and 20 min, respectively, in 10% Clorox® solution (Method #1, Table 1) and rinsed. Alternatively, seeds of *D. californica* and *S. leucophylla* were treated in concentrated H_2SO_4 for 4 min and 10 min, respectively, and rinsed (Method #2, Table #1). Following the last wash, seeds were poured (along with 1-2 ml of water) onto sterile filter paper in a sterile petri dish and transferred, with fine forceps or a bacterial loop, to 50 ml Erlenmeyer flasks (six seeds per flask, six flasks per treatment) containing 1) 5 ml of sterile H_2O (pH 5.0) or 2) 5 ml of liquid ½ strength Murashige and Skoog (MS) salts + MS vitamins + 20 g/l sucrose (Murashige and Skoog, 1962). The flasks were placed on a gyrotary shaker at 120 rpm under room temperature and lighting

conditions (diffuse light, 23°C ± 3°). All transfers were performed in a laminar flow hood. With the exception of the acid treatment for *D. californica*, experiments were repeated twice.

Germination Studies on Unimbibed, Cold-Imbibed, and GA_3-Stimulated Seeds of *D. californica* on Semi-Solid H_2O Agar Medium

Seeds were subjected to 3 treatments: unimbibed (Method #3, Table 1), imbibed (Method #4 Table 1), and imbibed with 6 mg/l GA_3 (Method #5, Table 1). Surface-disinfestation time was 10 min in H_2O_2. After disinfestation seeds were transferred (25 seeds each) to nine, 15 mm x 100 mm plastic petri dishes (3 per treatment) containing 25 ml of water agar. Incubation was at room temperature and room lighting conditions as described above. Observations were made daily until first germination was observed, then every other day for a period of 21 days. The experiment was repeated twice.

Germination of Seeds of *S. leucophylla*, *S. alata*, and *S. purpurea* Following Cold-Imbibition and Treatment In Concentrated H_2SO_4

Seeds of *S. leucophylla*, *S. alata*, and *S. purpurea* were prepared by method #6 (Table 1) and transferred (1 each) to individual wells of 12-well-plastic culture plates containing 3 ml of either sterile deionized H_2O or Phytomax Orchid Multiplication Medium (POMM, Sigma, St. Louis MO). Culture plates were sealed with Parafilm® (American National Can, Menasha, Wisconsin) and placed in a growth chamber at 27°C ± 2° with 16-hr d^{-1} illumination at 170 μmol m^{-2} sec^{-1}.

Effect of Scarification on Germination of Seeds of *D. californica*

The micropylar regions of 30 seeds of *D. californica* were removed with the aid of a dissecting microscope and fine pointed scalpel, and seeds were then subjected to Method # 3 (Table 1) and incubated at room temperature and light. The experiment was repeated twice.

Statistical Analysis

Results of sterile H_2O wash and surface sterilant treatments are reported as percent contaminated seeds after 15 days incubation on MYP. Treatments were compared using chi-square analysis with Yates correction (Schefler, 1979) of number of seeds contaminated after 15 days incubation and the number of seeds not contaminated. Data for germination in liquid medium were analyzed by converting number of seeds germinated after 21 days to percent. Percentages were transformed using arc sin transformation. T-tests were performed on the transformed data. Analysis of germination data from solid media experiments was performed with ANOVA followed by Fisher Post-Hoc tests. Comparisons of early and late germination between treatments were made with chi-square analysis of number of seeds germinated on day 15 vs. total number germinated after 21 days. ANOVA, t-test and transformations were performed with Statview 5.0 (Statview: Using Statview SAS Third Edition, 1999). Chi Square analysis was performed with a program provided by Dr. R. Koske (Dept. of Biological Sciences, URI).

RESULTS:

Surface Disinfestation – Sterile Wash

In all species washing with sterile water generally was ineffective for obtaining sterile seed material (Table #2). However, in some trials, surface disinfestation of seeds of *D. californica* was achieved with a sterile H_2O wash (Table 2, Trials 2, and 3).

Treatment in Surface Sterilants

Seeds of *D. californica* could be surface disinfested with all sterilants tested using exposure times as low as 4 min (Table 3). In contrast, seeds of *S. leucophylla* remained contaminated regardless of the treatment or exposure time (Table 3). Treatment of *S. leucophylla* in concentrated H_2SO_4 resulted in a consistent reduction in percent of contaminated seeds as exposure time in sterilant increased (Figure 1). A similar linear decrease in contamination was observed in response to treatment with Physan 20, but in much smaller increments, and the contamination rate remained high. Both 10% Clorox® and 3% hydrogen peroxide were inconsistent sterilants. Contamination sometimes spiked as time in sterilant increased (Fig.1). Chi –square analysis of treatment times for *S. leucophylla* seeds with concentrated H_2SO_4 suggested that optimum treatment time was between 8 and 12 min (Table 4).

Scanning Electron Microscopy

Dry seeds of *D. californica* are "tear-drop" shaped with finger-like trichomes on the seed surface (Fig. 2A). Erect trichomes (those with their long axis perpendicular to the long axis of the seed) were most numerous at the chalazal end of the seed (Fig. 2B) and diminished in density toward the tapering micropylar region

Seed coats of *S. purpurea*, *S. flava*, and *S. rubra* were similar to those of *S. leucophylla* in surface topography (Figs. 6A-H). In these species fungal hyphae, sporangia and spores were frequently observed on the surfaces of many of the seeds (Figs. 6i and j) or appeared to be growing out of damaged surface cells (Fig. 6J). In contrast, seeds of *Drosera* species showed considerable interspecific variation in surface topography (Figs. 7A-J). The surface of *Dionaea muscipula* seeds (Figs. 8A - H) was relatively smooth (Fig. 8A) with slight bulges in the outer periclinal wall of surface cells giving the seed coat surface a "cobblestone" appearance (Fig. 8B). The outermost layer of the seed coat was characterized by hollow, heavily sclerified cells (Fig. 8C) with thick anticlinal walls (Fig. 8D). The periclinal surface was covered by a thick, homogeneous, extracellular matrix (Fig. 8E). Although the seed coat layers appeared more impenetrable than in any of the other genera examined, evidence of fungal infection was observed (Figs. 8F and G). When viewed in paradermal section the interior of the seed coat had a honeycombed appearance (Fig. 8H).

Germination Studies in Liquid Medium

Germination of seeds of *D. californica* in water (Method #1, Table 1) was significantly greater than in ½ strength MS ($P=0.006$ and $P=0.004$respectively for two trials) at 21 days of incubation (Fig. 9). The number of seeds germinated on day 15 vs. the total number germinated by day 21 was significant at $P=0.01$ (chi square= 8.517) and $P=0.05$ (chi-square = 6.23) respectively for trials 1 and 2. Seeds of *D. californica* treated in H_2SO_4 (Method #2, Table 1) failed to germinate after 21 days of incubation.

Seeds of *S. leucophylla* could not be surface-sterilized in quantities required for further experiments. For instance, in one trial, contamination appeared early in 5 of 6 flasks containing 6 seeds each. Appearance of contamination sometimes followed germination and seeds germinated even in contaminated flasks (see Appendix A, Ancillary Results to Manuscript 1 for data on *S. leucophylla*). *S. leucophylla* showed 11.5% germination in H_2O after 21 days compared to no germination in liquid ½ strength MS in the only trial that produced usable data. Germination on solid ½ strength MS was not determined due to contamination in all flasks. Further work with *S. leucophylla* was limited to work with 3 seedlings that had germinated in one uncontaminated flask of 6 seeds in H_2O.

Germination on Semi-Solid H_2O Agar Medium

The response of seeds of *D. californica* varied widely between germination Methods #3, #4 and #5. The highest percent germination (~80%) was observed in seeds receiving cold-imbibition and GA_3 stimulation before germination (Fig. 10). All treatments differed significantly from each other (P=0.0005).

Germination of Seeds of *S. leucophylla*, *S. alata*, and *S. purpurea* Following Cold Imbibition and Treatment in Concentrated H_2SO_4

Seeds of *S. leucophylla*, *S. alata*, and *S. purpurea* failed to germinate after 60 days of incubation in a growth chamber with temperature and light conditions as previously described.

Effect of Scarification on Germination of *D. californica*

Germination of scarified seeds was 70% and 77% respectively after 21 days in two trials (data not shown- see Appendix A).

DISCUSSION

Surface Disinfestation

The very high effectiveness of the variety of sterilants tested for surface disinfestation of the seeds of *D. californica* was unexpected. SEM observations of the seed coat revealed a surface morphology that presented opportunity for the collection and adherence of spores of microorganisms. Also, seeds of *D. californica* have been reported as difficult to disinfest (Boulay, 1995). A lack of contamination during incubation of *D. californica* seeds was observed in preliminary experiments (data not shown). In addition, when data from the sterile wash series were collected, it became apparent that seed surfaces were either remarkably free of microorganisms or could be made free of them with a simple wash. The use of 3 different seed lots in these experiments suggests that this phenomenon is not seed-lot specific. Although all sterilants tested were effective, 10% Clorox® and 3% H_2O_2 were selected for use in subsequent experiments with *D. californica* because of their consistent effectiveness. Physan 20 was rejected after seeds disinfested in a 10% solution failed to germinate. Similarly, concentrated H_2SO_4 was rejected after seeds treated for 4 min failed to germinate (see Appendix A). SEM observations showed that seed coat integrity had been compromised by the acid.

In contrast, seeds of *S. leucophylla* were difficult to disinfest in quantity using the 4 sterilants tested. Although difficulty in disinfestation of explanted organ tissues from several species of carnivorous plants has been reported (Anthony, 1992; Crouch et al., 1990; Minocha, 1985), reports of difficulty in disinfestation of seeds used as initial explant material for culture of carnivorous plants has been limited (see Table 3,

Appendix A). The inability to effectively disinfest seeds of *S. leucophylla* may result from endophytes present within the seed coat or pathogens that gained entry during or after its maturation. SEM observations revealed fungal hyphae traversing the seed coat surface of *S. leucophylla* (Figs. 3F and I). Fungal hyphae and sporangia were also observed emerging from damaged cells of the seed surface of *S. purpurea* (Fig. 6J) and the micropylar region of the seed coat of *Dionaea muscipula* (Fig. 8E). This implies that they are not in the category of surface organisms, thus rendering surface sterilization insufficient as a disinfestation treatment for these seeds.

The effectiveness of H_2SO_4 as a disinfectant for seeds of *S. leucophylla* may have resulted from its ability to successively dissolve the seed coat layers (Figs. 3C-H) and reach previously sheltered endophytes or their spores. In preliminary experiments (data not shown) several species of *Drosera* and *Dionaea muscipula* were easily disinfested with 3% H_2O_2. Ease of disinfestation was most likely due to the unornamented outer layers of their seed coats as observed in SEM (Figs.7F and I; Fig.8A).

Contamination of explanted tissue of carnivorous plants has sometimes been attributed to the presence of endophytic organisms (Perica and Berljak, 1996; Anthony, 1992). Although the literature contains many reports of endophytes in non-carnivorous plants (e.g. White et al., 1993, 1986; Carroll, 1988; Hinton and Bacon, 1984), to my knowledge no investigations of endophytes in carnivorous plants have been reported.

Germination Studies

The rapid germination of unimbibed seeds of *D. californica* in H_2O and cold-imbibed seeds in H_2O agar is both surprising and fortuitous. Horticultural literature suggests that germination of seeds of *D. californica* requires a cold treatment of up to 2 months (Lecoufle, 1990; Pietropaolo and Pietropaolo, 1986). Earlier observations by Withner (1964) reported the failure of mature seeds of *D. californica* to germinate under laboratory conditions when sown immediately after collection; successful germination was only achieved through the use of immature seeds from green seed capsules. The research reported in this manuscript shows that cold imbibition of mature seeds of *D. californica* for 24 h in H_2O results in high germination percentages within 21 days. This rapid germination may be the result of the role of cold imbibition in breaking seed dormancy (Vidaver, 1977).

Seeds of *S. leucophylla, S. alata* and *S. purpurea* failed to germinate if cold imbibed prior to disinfestation in concentrated H_2SO_4. These results confirm observations by Ellison (2001). However, unimbibed seeds of *S. leucophylla* germinated when treated for 10 min in concentrated H_2SO_4 (see Appendix B) and incubated in H_2O.

Because sufficient numbers of seedlings can be produced without GA_3 stimulation, use of the hormone is not warranted for the production of seedlings for research because the effect of GA_3 on seedling morphology and its long-term effect on growth still require investigation. Similarly, because of the labor required, surgical removal of the micropylar pole of seeds was not considered to be worthwhile in establishment of this micropropagation system.

Summary

Based on the results of this investigation, protocols for the disinfestation and axenic germination of seeds of *D. californica* have been established. Additionally, this study indicates a possible relationship between seed coat morphology and ease of seed disinfestation.

Table # 1. Methods used for seed germination

Method #	Pre-Germinative Treatment			Sterilants for Surface Disinfestation**				Germination Medium***				
	Not imbibed	Imbibed*	Imbibed* +6mg/l GA_3	3% H_2O_2	10% Clorox	Conc. H_2SO_4	3x5min sterile washes	H_2O	H_2O agar	Liq. ½ Strength MS****	Solid ½ Strength MS****	POMM *****
1	X				X		X	X		X		
2	X					X	X	X		X		
3	X			X					X			
4		X		X					X			
5			X	X					X			
6		X				X	X	X				X
7		X		X					X			
8		X		X							X	

All seeds were stored unimbibed at 4-7°C prior to use in any treatment (experiments with each seed lot were conducted over a 3-4 month period).

* For 24 h in 10 ml H_2O +1 drop Tween 20 at 4-7°C

** 1 drop Tween 20 added to all sterilants except washes

*** Adjusted to pH 5.0

**** Murashige and Skoog (1962)

*****Phytomax Orchid Multiplication Medium (Sigma, St. Louis MO)

Table # 2. Effect of three 5 min washes in sterile H_2O on percent contamination of seeds incubated on MYP in the dark at 27°C.

Trial #	Species	# of Seeds	% Contamination	Days*
1	*Darlingtonia californica*	48	35.4	15
2			2.1	15
3			15	20 **
1	*Sarracenia leucophylla*	48	100	7
1	*S. purpurea*	48	94	15
1	*S. alata*	24	100	7
1	*S. flava*	24	75	15
1	*S. rubra*	24	2.1	15
2		24	100	15

- Experiments were run for 15 days, if all seeds contaminated earlier that time is noted.

** This experiment showed 15% contamination on day 15. No new contamination had occurred by day 20.

Table # 3. Percent contamination of unimbibed seeds* of *Darlingtonia californica* following treatment in four different surface sterilants.

Treatment	Time in sterilant and % contamination after 15 days incubation on MYP+ 10 g/l sucrose at 27°C in constant darkness			
	4 min	8 min	12 min	16 min
3% hydrogen peroxide	0	0	0	2.1
10% Clorox	0	0	0	0
Concentrated sulfuric acid	0	0	0	0
1.5% Physan	0	0	0	0
Sarracenia leucophylla				
3% hydrogen peroxide	65.0	100	50.0	44.0
10% Clorox	17.0	8.3	93.0	8.3
Concentrated sulfuric acid	52.1	40.0	14.6	6.3
1.5% Physan	91.7	7.8	67.0	63.0

* 48 seeds were used in each treatment

Table # 4. Seeds of *Sarracenia leucophylla*. Chi-square analysis of time in concentrated H_2SO_4 indicates optimum effective treatment time for disinfestation of seed surface lies between 8-12 min.

	8 minutes	12 minutes	16 minutes
4 minutes	NS	S (P=0.01)	S (P=0.01)
8 minutes		S (P=0.05)	S (P=0.01)
12 minutes			NS

S = significantly different
NS = not significantly different

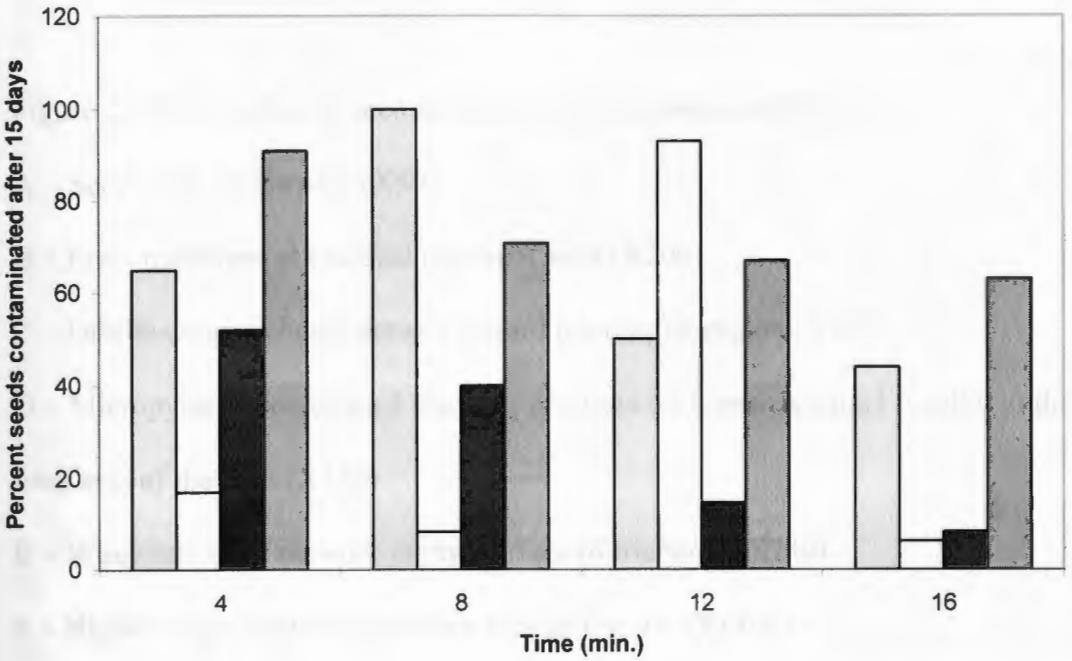


Figure 1. Effect of time in sterilant on percent contamination of seeds of *Sarracenia leucophylla after*15 days incubation on MYP medium in darkness at a constant temperature of 27°C. 3% H_2O_2 (gray bars); 10% Clorox (white bars); H_2SO_4 (black bars); 1.5% Physan 20 (dark gray bars)

Figure 2. SEM studies of seed surface of *Darlingtonia californica*

A – Seed of *D. californica* (X50)

B – Erect trichomes at chalazal region of seed (X200)

C – Diminishing trichome density toward micropylar region (X150)

D – Micropylar region of seed showing prostrate trichomes oriented parallel to the long axis of the seed (X150)

E – Waxy reticulate network on the surface of trichome (X780)

F – Higher magnification of surface seen in Fig. 1E (X1400)

G – Polka-dot appearance of seed surface (X180)

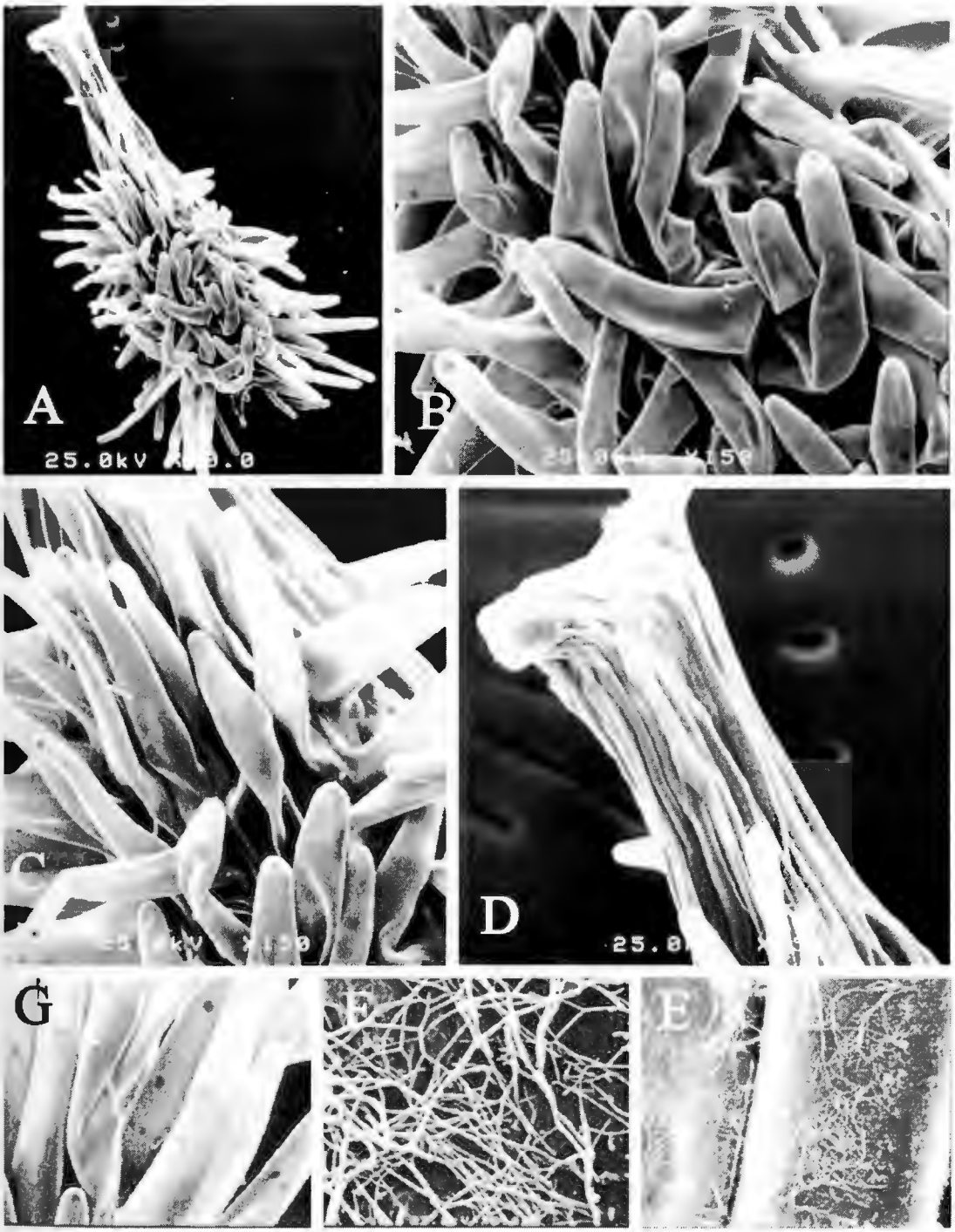

A
25.0kV
B
C
D
25.0
G
F
E

Figure 3. Seeds of *Sarracenia leucophylla* – Surface Topography

A- D – Seed surface morphology various orientations (X21)

E – Pitted appearance of surface (X50)

F – Collapsed periclinal walls of outer seed coat (X360)

G – Waxy covering of surface (X1900)

H – Waxy covering at higher magnification (X4000)

I – Fungal hyphae on seed surface (X300)

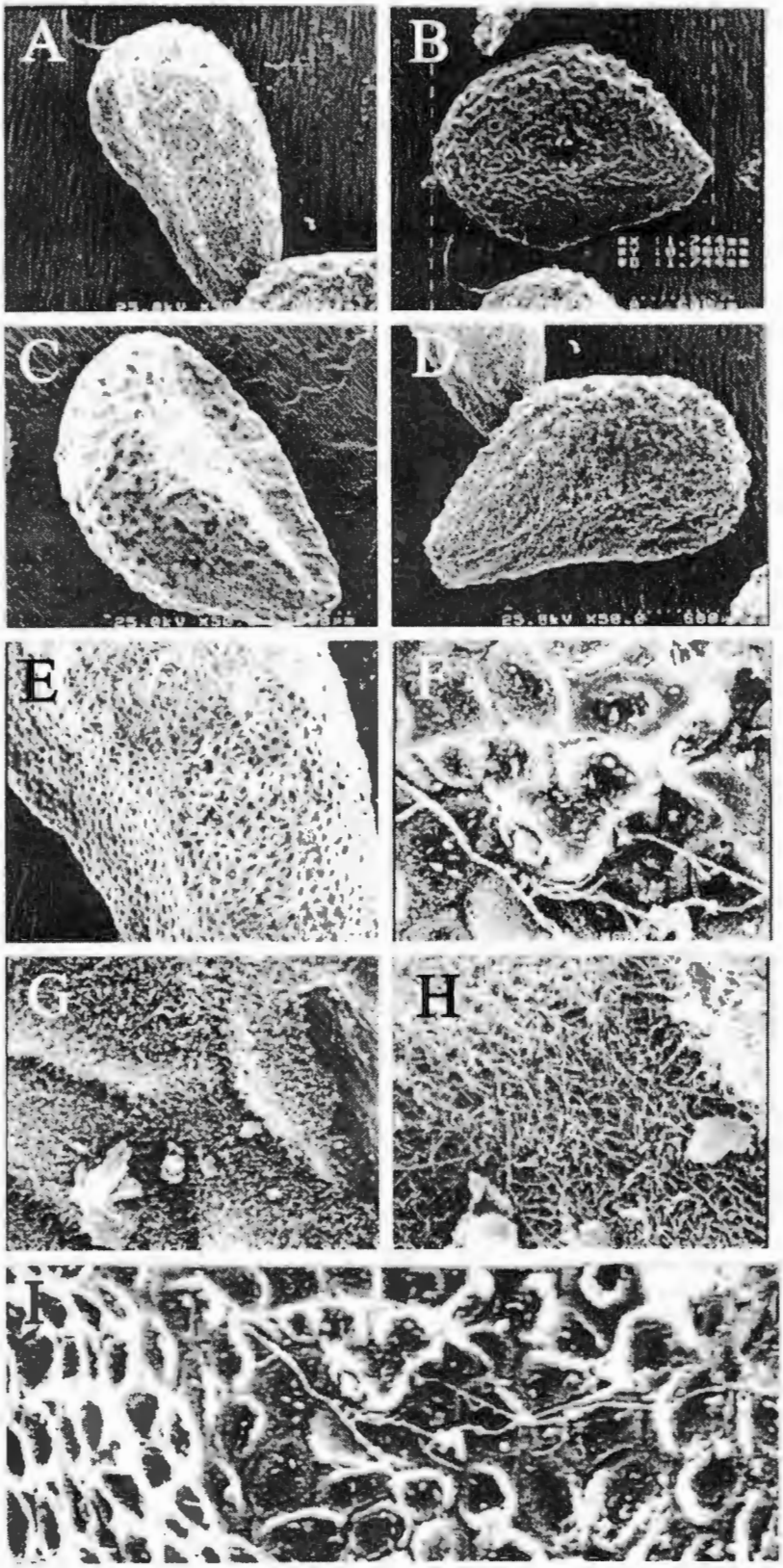

A
B
C
D
E
F
G
H
I

Figure 4. Seeds of *Sarracenia leucophylla* – Internal Structure

A – Multilayered coat at chalazal pole of seed and single layer parallel to long axis of the seed (X117)

B – Multilayered coat at micropylar pole showing hollow cells (X187)

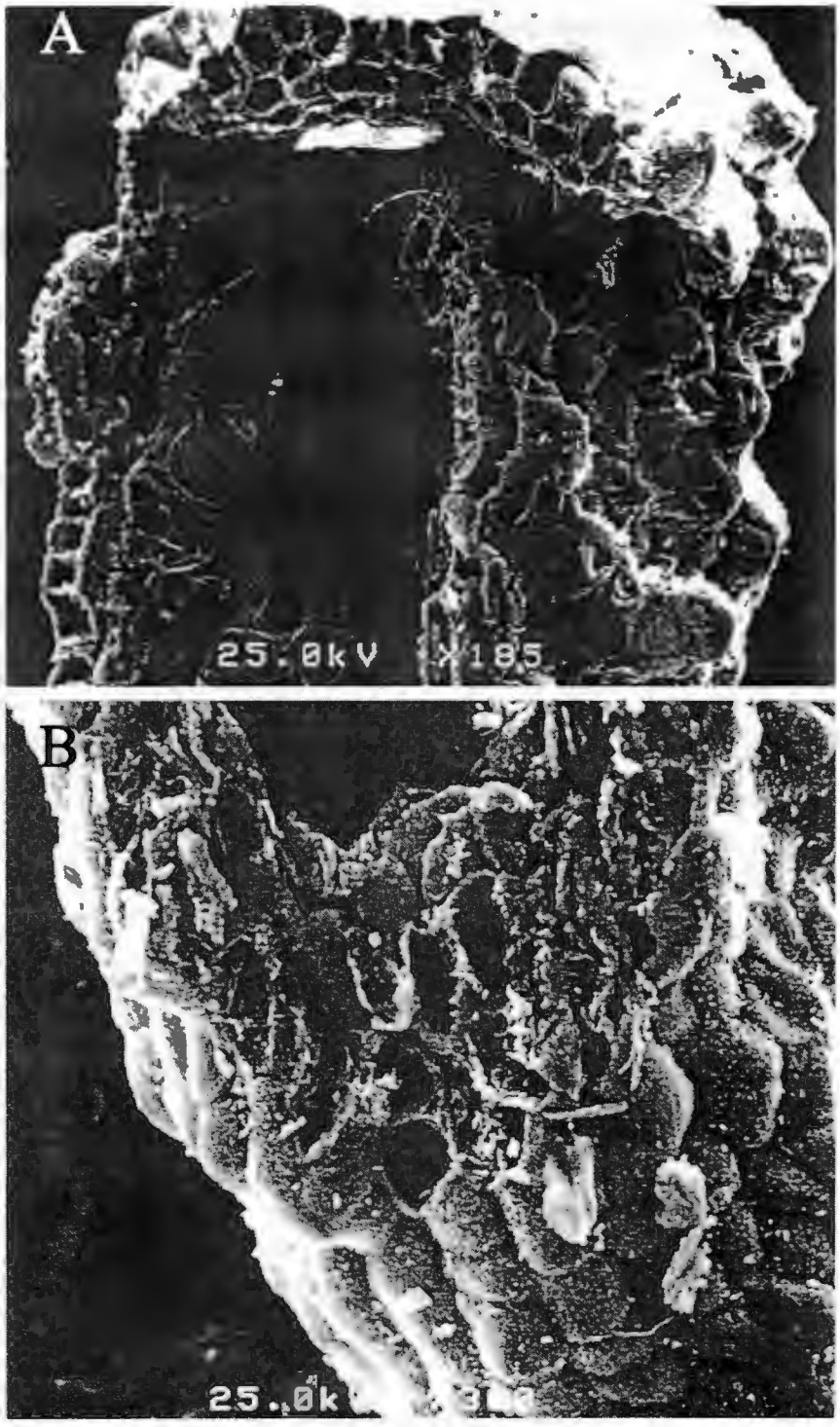

A
25.0kV X185
B
25.0k

Figure 5. Seed coats of *Darlingtonia californica* and *Sarracenia leucophylla* after acid treatments

A – *D. californica* 4 min. H_2SO_4 (X58)

B – *D. californica* with seed wall integrity compromised (X140)

C – *S. leucophylla* 4 min H_2SO_4 (X29)

D - *S. leucophylla* 4 min H_2SO_4 (X520)

E - *S. leucophylla* 8 min H_2SO_4 (X40)

F - *S. leucophylla* 8 min H_2SO_4 (X175 (cracks in seed coat occurred during SEM observation)

G - *S. leucophylla* 16 min H_2SO_4 (X26)

H - *S. leucophylla* 16min H_2SO_4 (X290)

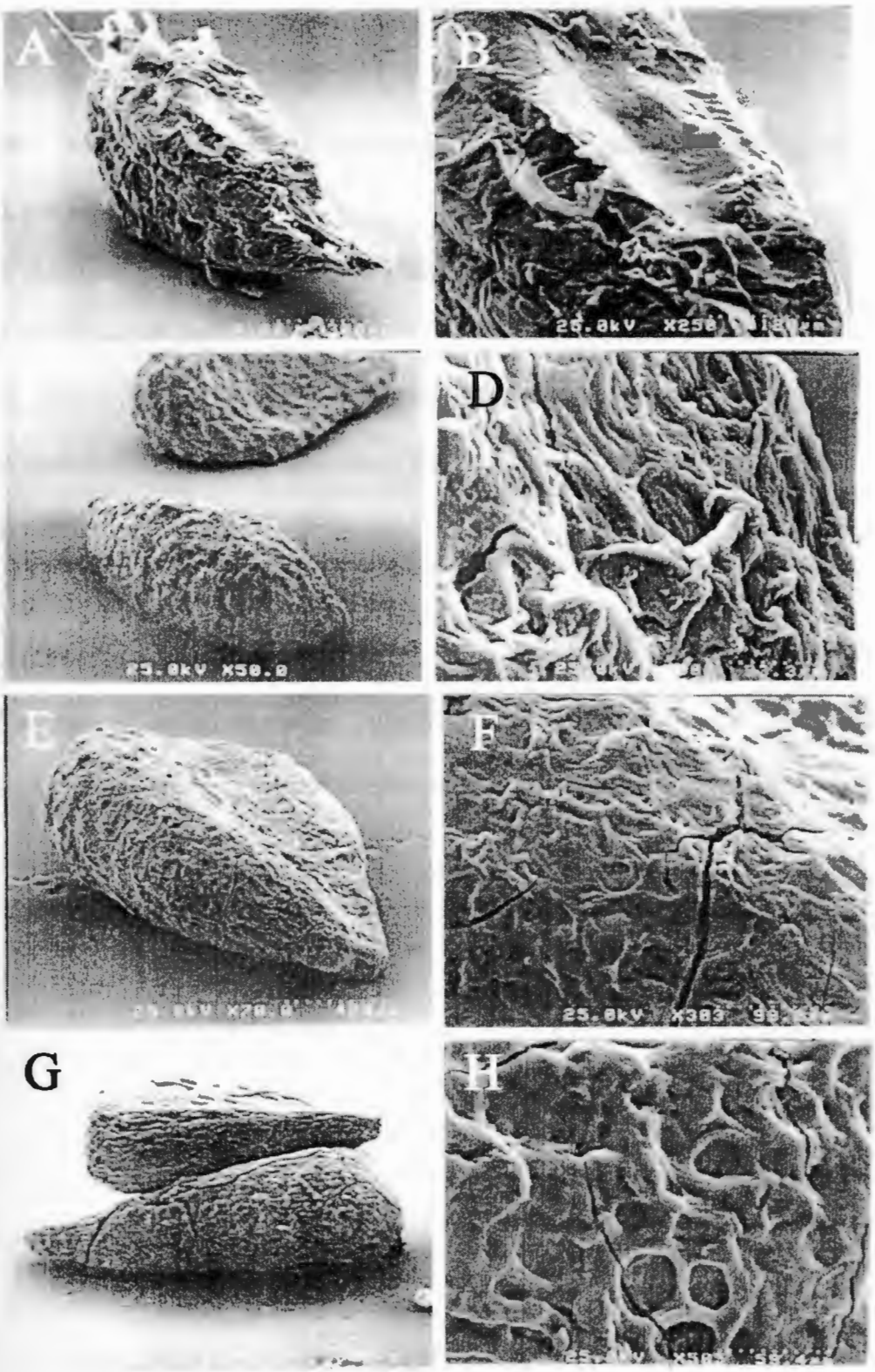

A
B
25.0kV X250
25.0kV X50.0
D
E
F
25.0kV X303
G
H

Figure 6. Seed surface topology of several *Sarracenia* species

A – *S. purpurea* seed (X23)

B – *S. purpurea* seed surface showing pitted appearance (X100)

C – *S. flava* seed (X28)

D – *S. flava* - Higher magnification of surface in 5C (X106)

E – *S. flava* – waxy covering of surface (X670)

F – *S. rubra* - seed (X72)

G – *S. rubra* seed (X170)

H – *S rubra* – waxy surface (X10,000)

I – *S. purpurea* – spores on surface (X2000)

J – *S. purpurea* – spores and hyphae emanating from damaged area (X650)

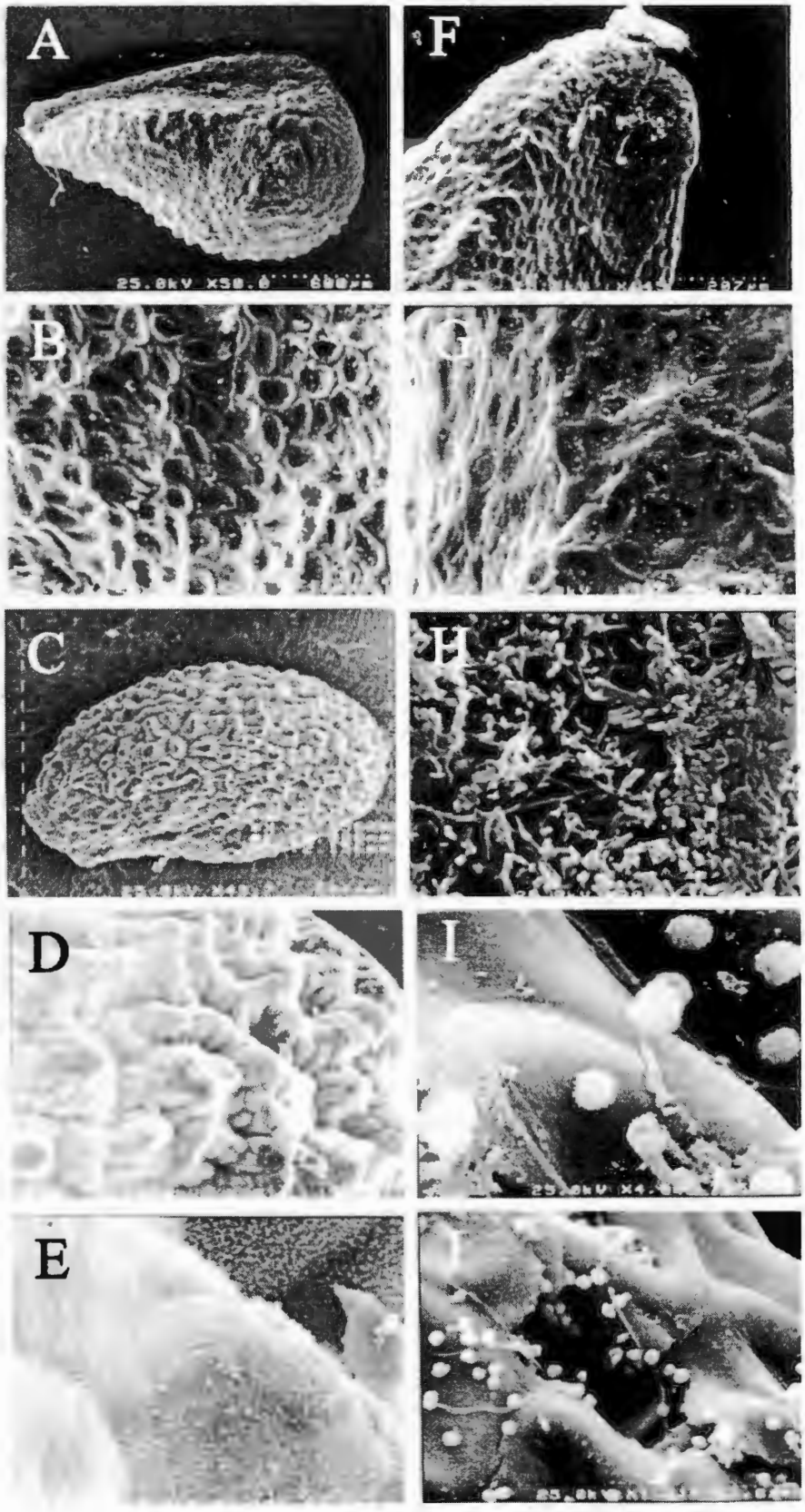

A
F
B
G
C
H
D
I
E

Figure 7. Seed surface morphology of several species of *Drosera*

A – *D. tracyi* – collapsed surface cells (X64)

B – *D. tracyi* – enlarged view of 1A (X590)

C – Seed of *D. intermedia* (X78)

D – *D. intermedia* – waxy covering of surface projections (X860)

E – *D. spathulata* seed (X40)

F – *D. spathulata* seed surface (X190)

G – *D. spathulata* (X1150)

H – *D. auriculata* seed (X40)

I – *D. auriculata* seed surface (X400)

J – *D. binata* seed (X57)

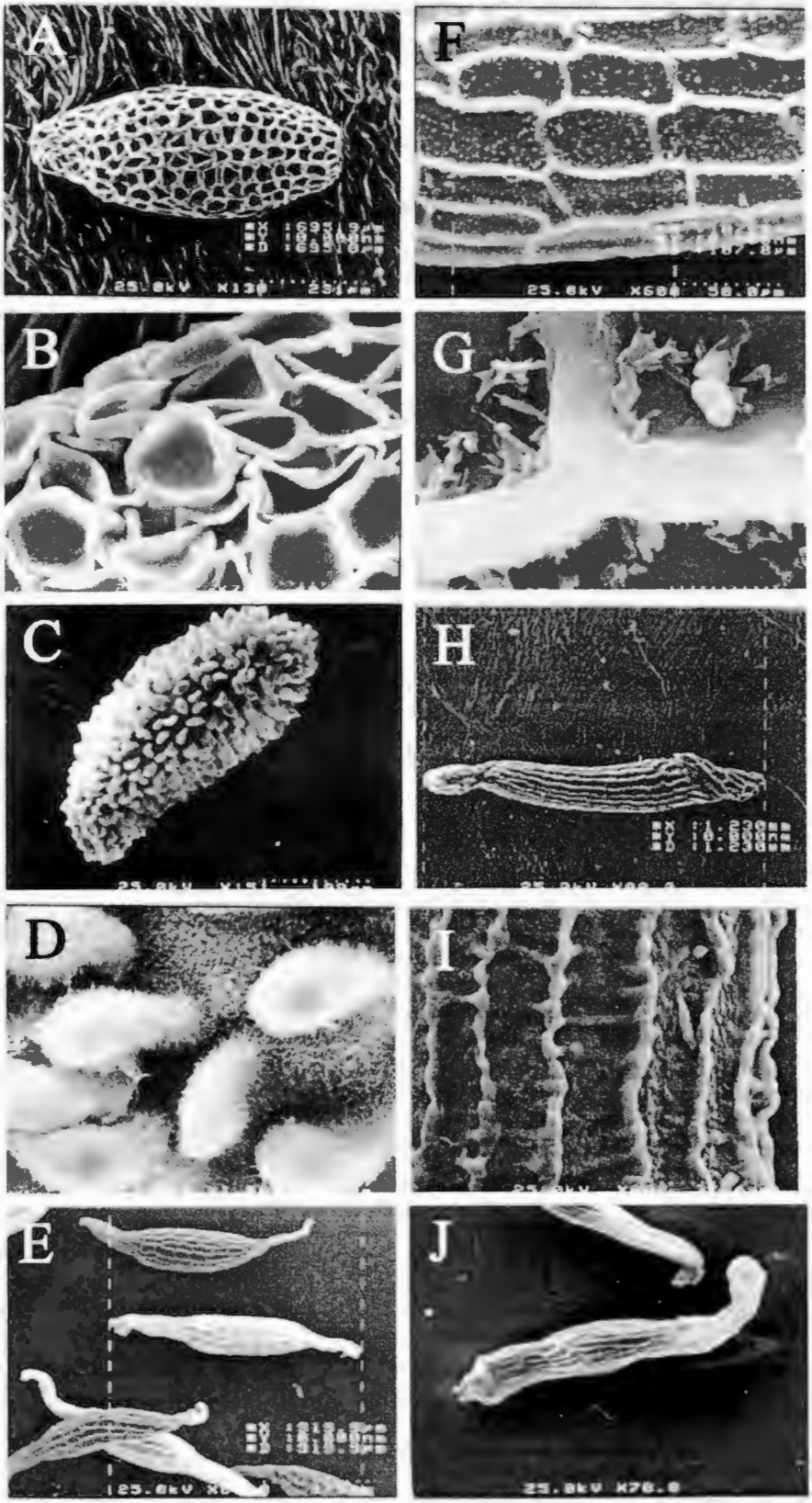

A
F
B
G
C
H
D
I
E
J

Figure 8. SEM study of seed surface topography and internal structure of seed coat of *Dionaea muscipula* (Venus Fly Trap)

A – Seed coat showing relatively smooth surface (X 150)

B – "Cobblestone" appearance of bulging periclinal walls (X320)

C – Hollow, heavily sclerified cells of outer coat (X280)

D – Thickened anticlinal walls of sclerified cells (X740)

E – Fungal hyphae with sporangia emerging from micropylar pole of a seed (X120)

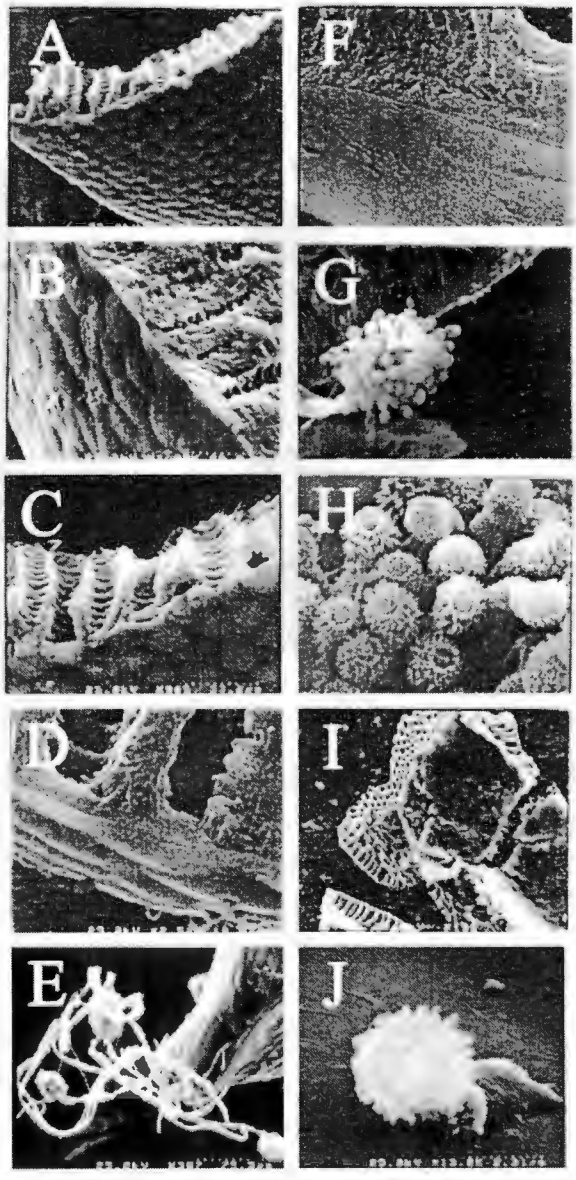
F – Thickened, homogeneous extracellular matrix (X3400)

G – Fungal sporangium (X630)

H – Fungal sporangium (X2800)

I – Honeycomb appearance of outer wall (X36)

J – Higher magnification of sporangium in figure 7E (X4100)


A
F
B
G
C
H
D
I
E
J

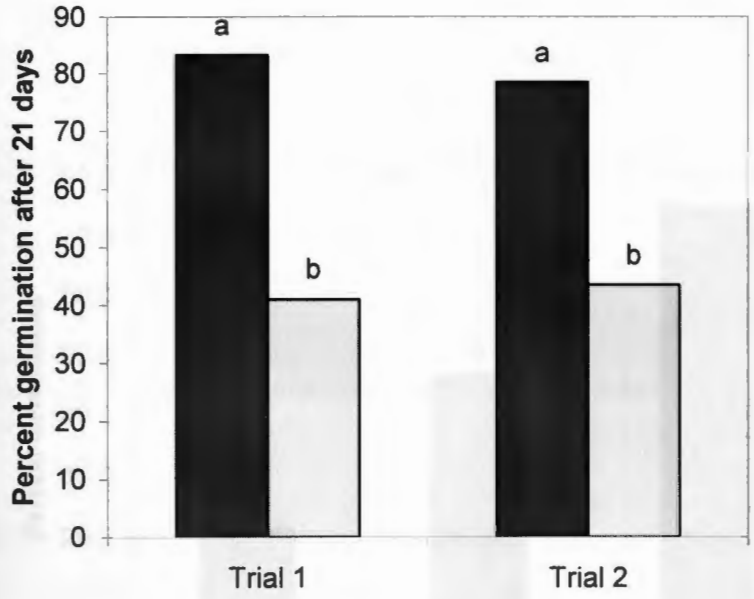


Figure 9. Comparison of percent germination of seeds of *D. californica* in H_20 (black) and liquid ½ strength MS medium (gray) in two trials. Columns denoted by different letters are significantly different. P=0.006 and P =0.004 in the two trials respectively.

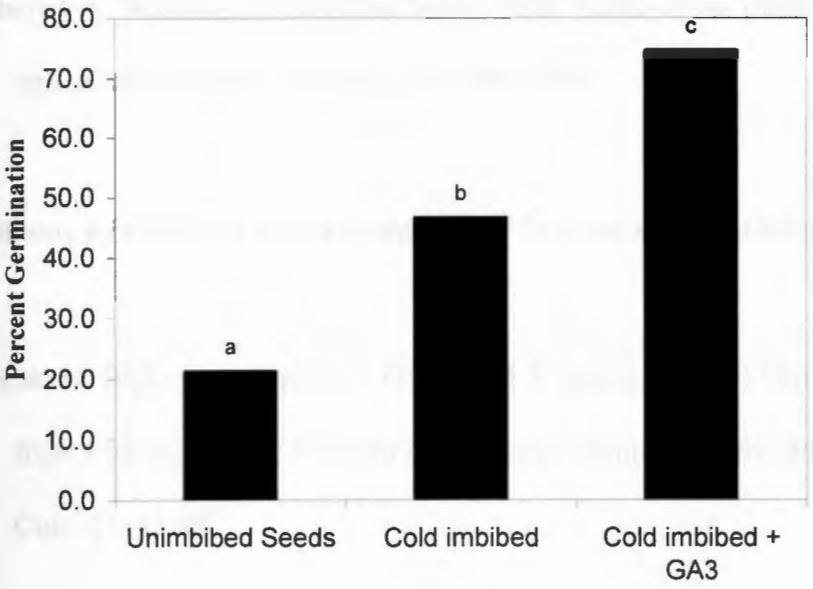


Figure 10. Effect of three different pre-germinative treatments on percent germination of seeds of *D. californica* after 21 days of incubation. Columns denoted by different letters are significantly different (Fisher's PLSD; P=<0.05). This experiment was repeated twice with similar results.

Literature Cited

Adamec L (1997) Mineral Nutrition of Carnivorous Plants: A Review. Bot. Rev. 63: 273-299

Albert VA, Williams SE & Chase MW (1992). Carnivorous plants: phylogeny and structural evolution. Science 257: 1491-1495

Anthony LJ (1992) *In vitro* propagation of *Drosera* spp. HortScience 27: 850

Bobak M, Blehova A, Kristin J, Ovecka M & Samaj J (1995) Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured *in vitro*. Plant Cell Tiss. Org. Cult. 43: 43-49

Bobak M, Blehova A, Samaj J & Ovecka M (1993) Studies of organogenesis from the callus culture of the sundew (*Drosera spathulata* Labill.). Plant Phys. 142: 251-253

Boulay J (1995a) Les plantes carnivores, essais de micropropagation. Bull. Acad. Soc. Lorraines Sci. 34: 151-159

Budzianowski J (2000) Naphthoquinone glucosides of *Drosera gigantea* from *in vitro* cultures. Plant. Med. 66: 667-669

Budzianowski J (1996) Naphthohydroquinone glucosides of *Drosera rotundifolia* and *D. intermedia* from *in vitro* cultures. Phytochemistry 4: 1145-1147

Budzianowski J (1995b). Naphthoquinones of *Drosera spathulata* from *in vitro* cultures. Phytochemistry 40: 1145-1148

Carroll G (1988) Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. Ecology 69: 2-9

Crouch IJ, Finnie JF & van Staden J (1990) Studies on the isolation of plumbagin from *in vitro* and *in vivo* grown *Drosera* species. Plant Cell Tiss. Org. Cult. 21: 79-82

D'Amato P (1998) The Savage Garden. Ten Speed Press. Berkeley CA

Ellison AM (2001) Interspecific and intraspecific variation in seed size and germination requirements of *Sarracenia* (Sarraceniaceae). Am. J. Bot. 88: 429-437

Givnish TJ, (1989) Ecology and evolution of carnivorous plants. Plant-Animal Interactions (pp. 243-290) McGraw-Hill, Inc. New York

Godt MJW & Hamrick JL, (1996) Genetic structure of two endangered pitcher plants, *Sarracenia jonseii* and *S. oreophila* (Sarraceniaceae). Am. J. Bot. 83: 1016-1023

Godt MJW & Hamrick JL, (1998) Allozyme diversity in the endangered pitcher plant *Sarracenia rubra* ssp. *alabamensis* (Sarraceniaceae) and its close relative *S. rubra* ssp. *rubra*. Am. J. Bot. 85 (6): 802-810

Gotsch SG & Ellison AM (1998) Seed germination of the northern pitcher plant, *Sarracenia purpurea*. Northeast. Nat. 5:175-182

Hinton DM and Bacon CW (1984) The distribution and ultrastructure of the endophyte of toxic tall fescue. Can. J. Bot. 63: 36-42

Hook ILI (2001) Naphthoquinone contents of *in vitro* cultured plants and cell suspensions of *Dionaea muscipula* and *Drosera* species. Plant Cell Tiss. Org. Cult. 67: 281-285

Koske RE (1977) Spiral growth of *Phycomyces*: some new observations in the Northern and Southern hemispheres. Mycologia 69: 189-193

Lecoufle M (1990) Carnivorous Plants: Care and Cultivation. Cassell Publishers Ltd. London, UK

Lichtscheidl IK, Lancelle SA & Hepler PK, (1990) Actin-endoplasmic reticulum complexes in *Drosera*. Protoplasma 155: 116-126

Lloyd EF (1942) The Carnivorous Plants. Chronica Botanica Co. Waltham, MA

Mandossian AJ, (1966) Germination of seeds in *Sarracenia purpurea* (pitcher plant). Mich. Bot. 5: 67-69

Miles DH, Kokpol U, Zalkow LH, Steindel SJ & Nabors, JB (1974) Preliminary investigation of antitumor activity of *Sarracenia flava*. J. Pharm. Sci. 63: 613

Minocha SC (1985) *In vitro* propagation of *Dionaea muscipula*. HortScience 20: 216-217

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497

Perica MC & Berljak J (1996) *In vitro* growth and regeneration of *Drosera spathulata* Labill. on various media. HortScience 31: 1033-1034

Pietropaolo J & Pietropaolo P (1986) Carnivorous Plants of the World. (pp. 25-73) Timber Press, Portland , Oregon

Samaj J, Bobak M, Blehova A, Kristin J & Auxtova-Samajova O (1995) Developmental SEM observations on an extracellular matrix in embryogenic calli of *Drosera rotundifolia* and *Zea mays*. Protoplasma 186: 45-49

Schefler WC (1979) Statistics for the Biological Sciences. Second Edition. Addison-Wesley Publishing Company. Reading MA

Schnell DE (2002) Carnivorous Plants of the United States and Canada. Second Edition. Timber Press. Portland, Oregon Pgs. 229-242

Slack A (2000) Carnivorous Plants. MIT Press. Cambridge MA

Vidaver W (1977) Light and seed germination. In: The Physiology and Biochemistry of Seed Dormancy and Germination. Khan AA (ed) North Holland Publishing Company, Amsterdam

White JF, Jr., Anthony GE & Chandler KF (1993) Endophyte-host associations in grasses. XVIII. Moisture relations and insect herbivory of the emergent stromal leaf of *Epichloe*. Mycologia 85: 195-202

White JF & Cole GT (1986) Endophyte-host associations in forage grasses. IV. The endophyte of *Festuca versuta*. Mycologia 78: 102-107

Withner CL (1964) Notes on the cultivation of insectivorous plants *in vitro*. Bull. Torr. Bot. Club 91: 412-413

Zenk MH, Furbringer M & Steglich W (1969) Occurrence and distribution of 7-methyljuglone and plumbagin in the Droseraceae. Phytochemistry 8: 2199-2200

ESTABLISHMENT OF A MICROPROPAGATION SYSTEM FOR MEMBERS OF THE SARRACENIACEAE

II.

IN VITRO GROWTH AND EARLY SEEDLING DEVELOPMENT OF THE NORTH AMERICAN PITCHER PLANT *DARLINGTONIA CALIFORNICA* TORREY

ABSTRACT

A simple effective system for *in vitro* growth, multiplication and rooting of axenically germinated seedlings of *D. californica* has been developed. Seedlings grown on solid ½ strength Murashige and Skoog medium produced more biomass and more and longer pitcher leaves than seedlings grown on other solid media assayed. Root development on all solid media was minimal and usually limited to the seminal root. Seeds stimulated by gibberellic acid prior to germination and exposed to auxin and cytokinin during early seedling development produced multiple offshoots as well as fibrous root systems when transferred to ½ strength liquid medium containing charcoal. Similarly treated seedlings transferred to ½ strength liquid media without charcoal produced multiple offshoots but fewer roots. Seedlings cultured in medium without charcoal produced more but smaller pitchers than seedlings cultured in medium containing charcoal. Multiplication did not occur on solid media, and seedling growth was stunted. Seedling multiplication through offshoots occurred in all liquid media and was both prolific and rapid.

INTRODUCTION

Manuscript 1 (this dissertation) reported techniques for the effective surface sterilization and subsequent axenic germination of seeds of *Darlingtonia californica*. This manuscript reports on the selection of a suitable growth and rooting medium for *in vitro* germinated seedlings that also allows the induction of clonal multiplication. Growth and rooting were investigated using both solid and liquid media.

Darlingtonia californica is a North American pitcher plant naturally occurring in Oregon and California (Schnell, 2002). It is interesting as a study organism not only because of its carnivorous habit but also because it offers the opportunity to study a combination of unique developmental and physiological events. For instance, *D. californica* usually grows beside cold (below 20°C) running streams in serpentine soils containing levels of nickel, zinc, chromium and other metals that are phytotoxic to many plant species (Schnell, 2002). However, it accumulates only low levels of these metals (Reeves et al., 1983). Additionally, it forms two morphologically and anatomically different pitcher types from the same meristem during the course of its development (Frank, 1975). Such a developmental switch offers the opportunity for investigations at both the cytological and genetic levels. Future research utilizing *D. californica* as a study organism will require the availability of sufficient amounts of plant material. This may be difficult because commercially produced adult or juvenile plants are seldom easy to obtain. Successful *in vivo* greenhouse cultivation has been limited to a few private collections (Schnell, 2002).

Although *D. californica* has been the subject of several histological and developmental investigations for over a century (see Lloyd, 1942 for early references) little work has been done on its culture *in vitro*. Withner (1964) reported a short series of qualitative observations on the application of orchid tissue culture techniques to carnivorous plants. However, he reported the failure of *D. californica* to form *roots in vitro* after two trials. Also, *D. californica* has been included as part of a broad feasibility survey on the micropropagation of several carnivorous plant genera (Boulay, 1995). Although Boulay (1995) reported that few roots were formed *in vitro*, results were not reported quantitatively.

This manuscript reports on the establishment of a system for *in vitro* culture of *D. californica* that provides a continuing source of explant material for subculture and future experimentation.

MATERIALS AND METHODS

Plant Material

For growth studies on solid media, seeds of *D. californica* were surface disinfested and germinated using Method # 2, Manuscript 1 (this dissertation). For growth studies in liquid media, seeds of *D. californica* were imbibed at 4-7°C in 10 ml of sterile deionized H_2O (pH 5.0) with one drop of Tween 20. After 24 h the H_2O was replaced with 10 ml of an aqueous solution of GA_3 (6 mg/l), and imbibition was continued for 12 days at 4-7°C. Seeds were then surface disinfested in 3% H_2O_2 (drugstore-variety, CVS®) with one drop of Tween 20 for 12 min, transferred (without rinsing) to two 250 ml Erlenmeyer flasks containing 75 ml of Phytomax Orchid Multiplication Medium (POMM; see Table 1 for medium components;pH 5.0). Flasks were incubated in a growth chamber at 27°C ± 2° with 16-hr d^{-1} illumination at 170 µmol m^{-2} sec^{-1}.

Growth Studies on Solid Media

Three basal media were used: MS (Murashige and Skoog, 1962) at ½ strength salts, Burgeff's N_3f (Arditti, 1982), and a modified, sphagnum-based medium (Withner, 1964). Media components are listed in Table #1. MS medium was purchased pre-mixed from Sigma. Burgeff's N_3f medium and sphagnum- based medium were prepared in the laboratory. For sphagnum based-medium, freshly collected live, unwashed, green sphagnum was cleaned of leaf litter and other debris then compacted in a 500 ml beaker. The compacted moss was transferred to a household blender and finely blended for 3 min in 500-700 ml of deionized water. Salts and organics were added before bringing the sphagnum solution to volume (1000

ml). All media contained 20 g/l sucrose as the carbon source and 1 ml of MS vitamin solution (1000X, Sigma). The pH of all media was adjusted to 5.0 with KOH or HCl prior to the addition of agar. Agar was melted by heating the solution on a hot plate with constant stirring, poured into a graduated dispensing column and aliquots of 15 ml were dispensed into 125 mm x 25 mm test tubes. Tubes were capped with translucent plastic closures and autoclaved (121°C for 15 min).

In a laminar-flow hood, 2-3 week old seedlings were transferred, individually, to tubes of medium (18 tubes per treatment). Tubes were sealed with Parafilm and placed in a growth chamber with temperature and light conditions as previously described. After 12 weeks of culture the plants were harvested and data collected. The parameters examined were the number of pitcher leaves per seedling (pitchers less than 3 mm in length were not scored), length of pitcher leaves (measured to the nearest mm), and total dry weight of explants per treatment. For dry weights, plants were dried at 60°C for two days and then weighed. Qualitative observations regarding color and general vigor were recorded. Experiments were repeated twice.

Growth and rooting in liquid media

Triplicate 250 ml Erlenmeyer flasks containing 75 ml of 1) ½ strength MS, 2) ½ strength MS with 2 g/l activated charcoal (Sigma, St. Louis, MO), 3) ¼ strength MS or 4) ¼ strength MS with 2 g/l activated charcoal were each inoculated with 5 seedlings with offshoots (9-12 pitcher leaves) that had been grown on solid POMM for 2 months. All media were adjusted to pH 5.0 prior to autoclaving.. Concentrations of sucrose and vitamins were as described for solid media. Flasks were transferred to a

growth chamber with light and temp conditions as described above. After 6 ½ weeks of culture, plants were harvested and data collected.

Statistical Analyses

Growth on Solid Media

Comparisons of numbers of pitchers produced per seedling between different media formulations were made using ANOVA followed by Fishers Post Hoc test. For comparison of pitcher length, average pitcher lengths per replicate were log transformed then analyzed as described above. Dry masses are reported as percent difference between treatments.

Rooting and Growth in Liquid Media

To determine average pitcher lengths, the longest 25 pitchers in each flask were measured to the nearest mm, and average pitcher length per flask was calculated. In assessing the total number of pitchers per flask, only pitchers longer than 2 cm were measured. Vitrified or etiolated pitchers were not scored. Total number of roots per flask was determined by scoring the number of roots observed on 17 separate clusters of pitchers (each cluster arising from a single rhizome). Dry weight of total plant material from each flask was obtained as described previously. Differences among treatments were analyzed with ANOVA and Fisher's Post Hoc test.

RESULTS

Growth on Solid Media

Seedlings grown on solid ½ strength MS medium produced more pitchers than those grown on either Burgeff's N_3f medium (P = 0.002), or on sphagnum based medium (P= 0.002, Fig. 1). Although growth of seedlings on Burgeff's medium resulted in the least number of pitchers produced, this difference was not significant compared with those grown on sphagnum medium. The average length of pitchers was significantly greater for seedlings grown on MS medium than those grown in sphagnum medium (P= 0.03) but not significantly different from those grown in Burgeff's medium (Fig. 2). Qualitatively, seedlings grown in ½ MS medium appeared greener and healthier than those grown in the other media formulations (Fig. 3A). Seedlings grown in Burgeff's medium (Fig. 3B) began to exhibit a yellow appearance soon after transfer from H_2O agar. Several began to brown by the end of the experiment, and growth appeared to be arrested. In contrast to seedling growth on both ½ MS and Burgeff's, seedlings grown in sphagnum-based medium (Fig. 3C) occupied an intermediate position and seedling color ranged from green to yellow green. Except for the seminal root, roots were not observed in any of the media tested. In general, growth was slow on all three media. Averages of two trials for the collective dry weights of all seedlings in a treatment were 0.057 g (½ MS), 0.020 g (Burgeff's) and 0.019 g (sphagnum).

Rooting and Growth in Liquid Media

Production of new pitchers was both rapid and prolific in all liquid formulations tested (Fig. 4). Clusters of pitchers could be separated (Fig. 5A) and

were observed to originate from a single central rhizome. The large number of pitchers produced in liquid culture was in stark contrast to that produced from the single seedlings grown on solid ½ strength MS medium for six months (Fig. 5B). The number of pitchers was significantly greater without charcoal in either ½ strength or ¼ strength liquid MS medium ($P<0.02$), but was not affected by medium strength in the presence or absence of charcoal ($P>0.93$, Fig. 6). In contrast, pitcher length was significantly greater with charcoal in either ½ and ¼ strength MS ($P <0.0001$, Fig. 7). Pitchers produced in medium containing charcoal were brighter green and appeared to be more robust due to an observed (but not quantified) difference in diameter. Root production was greater in ½ strength medium with charcoal than in any other treatment ($P=0.0001$, Fig. 8). However, significantly more roots were produced in ½ strength MS without charcoal than in ¼ MS with or without charcoal ($P=0.01$ and $P=0.005$). Roots formed in ½ strength MS without charcoal were sometimes very long, but multiple root formation was observed only with charcoal (Figs. 9A-D). Roots were usually absent in ¼ strength MS without charcoal (Fig. 10a) and were solitary and short when present (Figs.10B and D). Addition of charcoal to ¼ strength MS produced longer solitary roots (Fig. 10C).

Charcoal did not affect the dry weights of plant tissues produced after 6½ weeks of growth in ½ strength MS medium ($P=0.95$, Fig. 11). However, both ½ strength treatments were significantly different from ¼ strength MS liquid medium with and without charcoal ($P< 0.04$).

DISCUSSION

The object of this study was to select a suitable medium for *in vitro* growth and rooting of *D. californica*. The three media assayed were chosen from the literature based on results reported from prior use in related studies. MS medium at ½ strength salts was selected because reduced-salt MS medium is commonly employed for *in vitro* culture of many carnivorous plant species (see Appendix B). Withner (1964) used Burgeff's N_3f medium in his studies on *D. californica*. However, the formulation is not reported and was obtained from Arditti (1982). The components for sphagnum-based medium were based on Withner (1964). Potassium nitrate and myo-inositol concentrations for sphagnum-based medium were based on full strength MS medium (Murashige and Skoog, 1962).

Media were chosen based on preliminary studies (sphagnum-based medium) or results of previous studies (Burgeff's N_3f medium, Withner, 1964). However, the growth of *D. californica* was poor on all solid media tested. Of the media tested, sphagnum-based medium was the least defined. The condition of the sphagnum used in preparation of the medium and the conditions present in the habitat where it was collected, may have affected levels of macro and micronutrients available in the final medium formulation. This may have affected the growth promoting and growth sustaining ability of the medium because certain plants have requirements for specific concentrations of micronutrients (Dodds and Roberts, 1995). Vitamin and sucrose levels were the same in all media. However, nitrogen was supplied as potassium nitrate and casein hydrolysate in sphagnum-based medium, rather than ammonium ion, as in the other media. Growth of plants or plant tissue in culture has been shown

to be most rapid when both nitrate and ammonium are available (George and Sherrington, 1984). Similarly, the poor growth on solid Burgeff's medium may have resulted from a deficiency in micronutrients, not present in published formulations (Withner, 1964, Arditti, 1982) or media prepared for this study. Withner reported that casein hydrolysate enhanced growth of *D. californica* by 40% in liquid Burgeff's medium. In the present experiments casein hydrolysate was not added to the formulation.

Growth on solid medium, regardless of the formulation, may have been poor because *D. californica* did not form roots and was therefore unable to absorb enough water or nutrients to allow more vigorous growth. Also, the seminal root may have depleted nutrients in the media surrounding it due to the relatively small volume of media in the tube. In contrast, growth on ½ strength MS may have been greater than on the other formulations because of its completeness in terms of macro and micronutrients.

One possibility for the prolific growth of *D. californica* in liquid medium at both ½ and ¼ strength salts may have been the availability of nutrients to the pitcher leaves. Because of its carnivorous habit, internal zones of the pitcher are specialized for absorption of nutrients from digested prey (Lloyd, 1942). In a sense, the liquid medium may have served, literally, as a nutrient soup for the pitcher leaves. Additionally, incubating plant material in liquid-shaken cultures has been shown to increase the rate of shoot proliferation in some species of non-carnivorous plants (Hu and Wang, 1983).

Increased pitcher production without charcoal in ½ and ¼ strength MS media may be related to the endogenous production of hormones which stimulate proliferation of *D. californica* in its natural habitat. In media containing charcoal phytohormones may have been adsorbed before they could reach levels that stimulate production of new pitchers. Activated charcoal added to media has been shown to adsorb cytokinin (Takayama and Misawa, 1980). Because the seedlings used to initiate this experiment had been exposed to GA_3 and grown on medium containing phytohormones, carry over could have had the ongoing effect of enhancement of pitcher production in charcoal free medium. However, the effect of any carry over of phytohormones to media with charcoal may have been negated due to adsorption of the hormones by the charcoal. In media containing charcoal, pitcher length may have been greater due to adsorption of phytohormones allowing resources to be allocated to the growth of individual pitchers instead of the production of new pitchers.

Increased root production in ½ MS with charcoal in comparison to all other treatments, could have been due to greater nutrient availability in the ½ MS. Absorption of nutrients by pitcher leaves of carnivorous plants has been shown to stimulate uptake of nutrients by roots and contribute to greater root length (Adamec, 2002). Additionally, activated charcoal may adsorb toxic substances in the medium resulting in increased root production (Ziv, 1979; Takayama and Misawa, 1980). In the present study, the effect of charcoal on root induction appears to be synergistic with the strength of the medium. Charcoal as an additive for root induction has been used for *in vitro* rooting of difficult-to-root carnivorous plant species such as

Nepenthes (Redwood and Bowling, 1990). More work is required in order to clarify the role of charcoal in root induction.

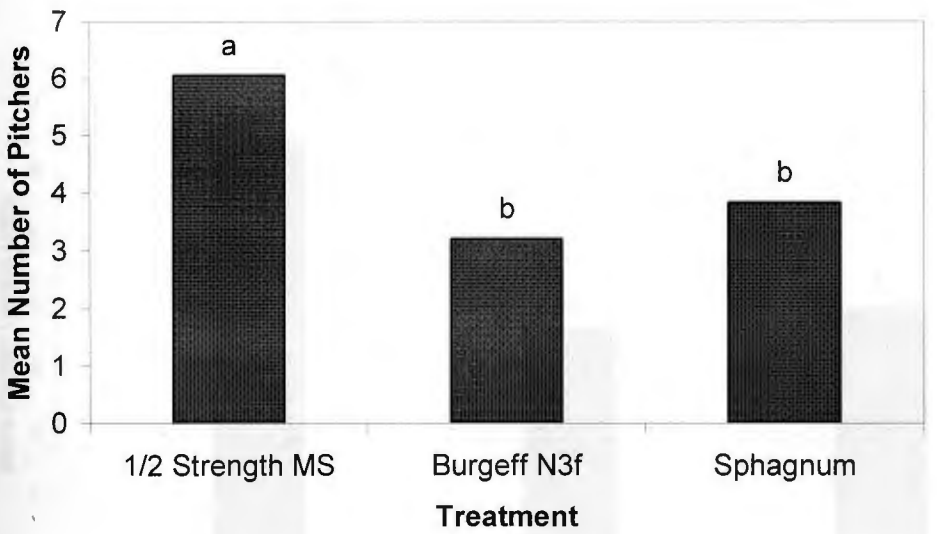


Figure 1. Effect of medium on number of pitchers produced by seedlings of *Darlingtonia californica* after twelve weeks of growth on three different solid media. Treatments denoted by different letters were significantly different. (Fisher's PLSD; P=<0.05). Intra-treatment differences were not significant.

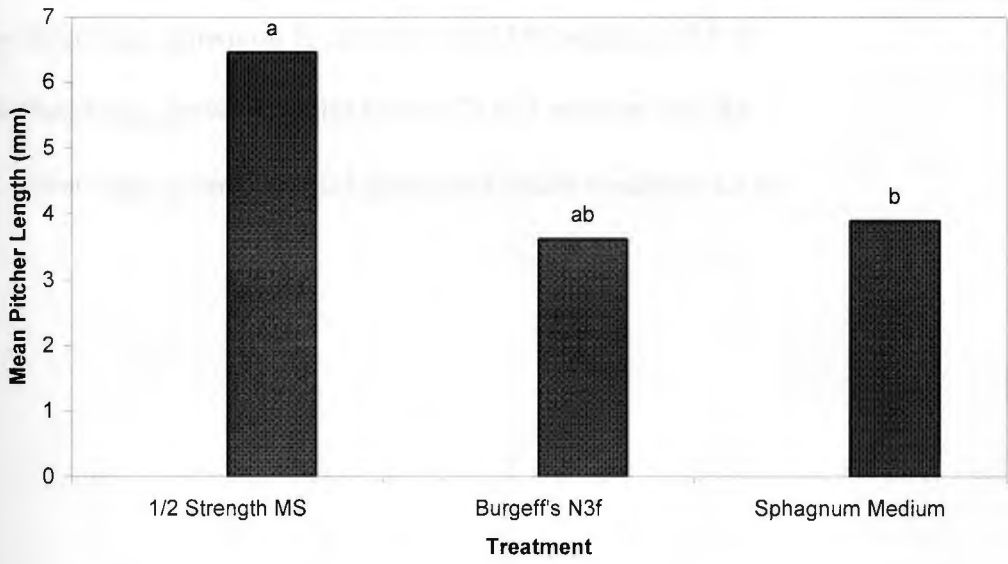


Figure 2. Effect of medium on length of pitchers produced by seedlings of *Darlingtonia californica* after twelve weeks growth on three different solid media. Treatments denoted by different letters were significantly different. (Fisher's PLSD; P=<0.05)

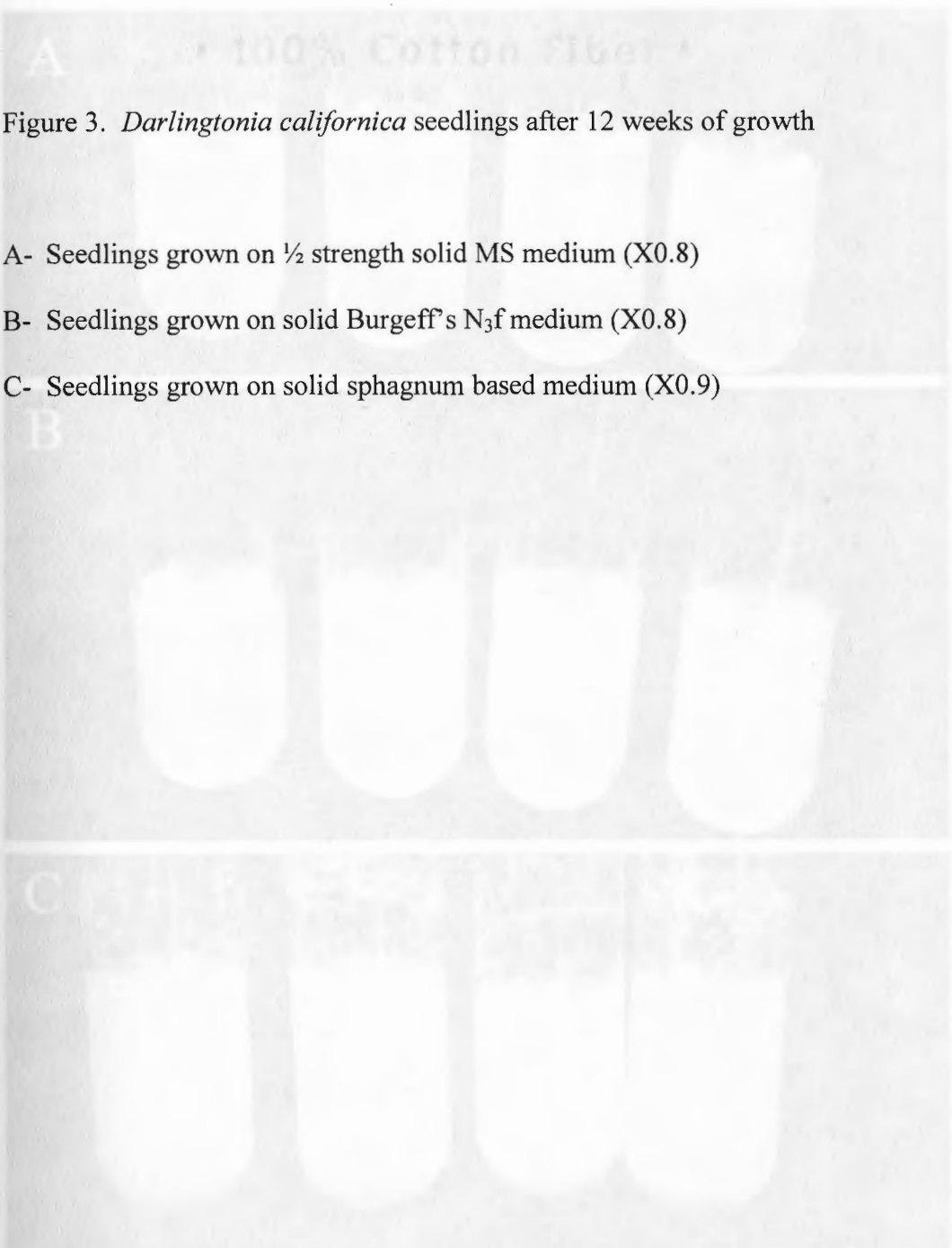


Figure 3. *Darlingtonia californica* seedlings after 12 weeks of growth

A- Seedlings grown on ½ strength solid MS medium (X0.8)

B- Seedlings grown on solid Burgeff's N_3f medium (X0.8)

C- Seedlings grown on solid sphagnum based medium (X0.9)

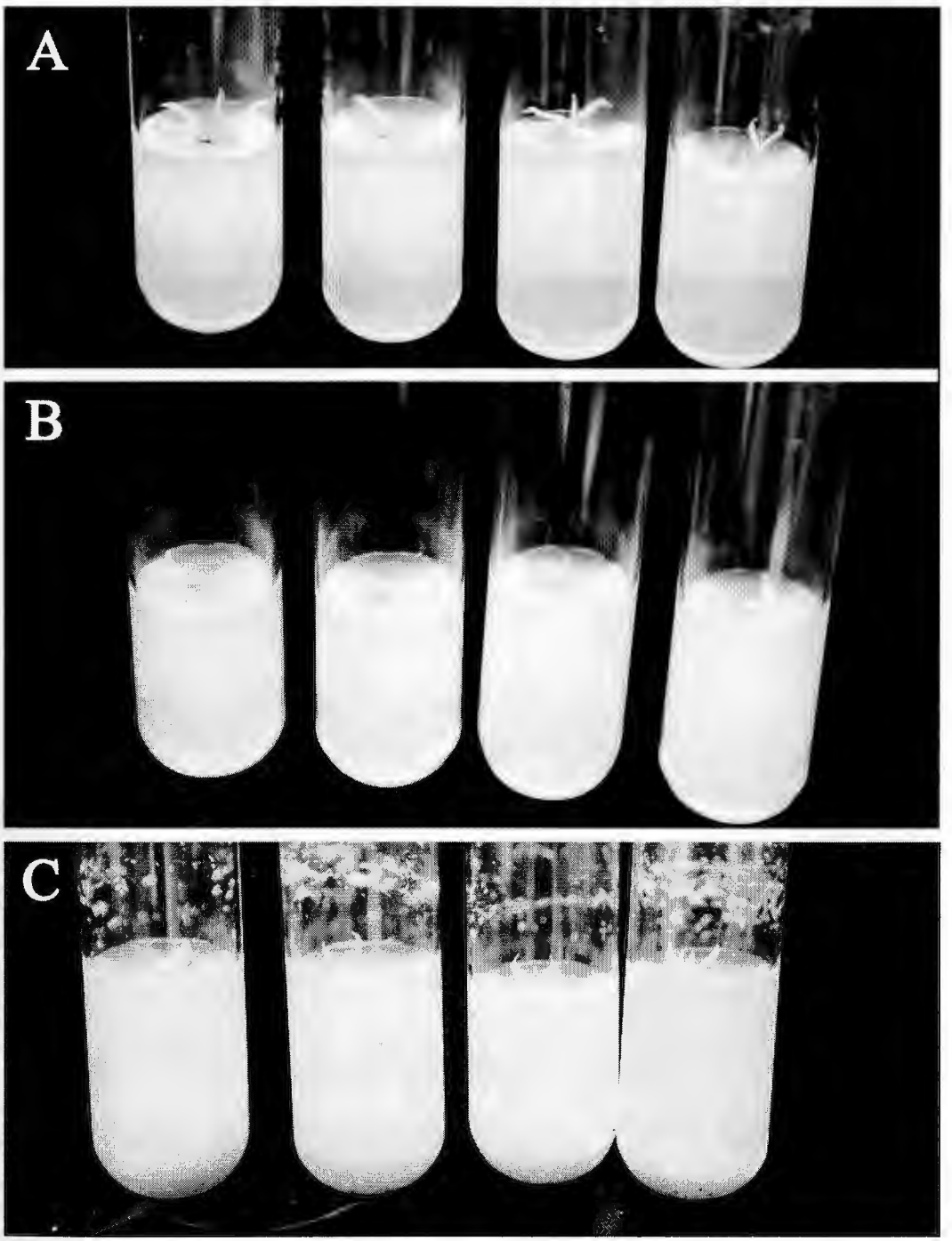

A
B
C

Figure 4. *Darlingtonia californica* seedlings after 6 1/2 weeks of growth in liquid media

A – Seedlings grown in liquid ½ strength MS medium with charcoal (X0.6)

B – Seedlings grown in liquid ½ strength MS medium without charcoal (X0.7)

C – Seedlings grown in liquid ¼ strength MS medium with charcoal (X0.6)

D – Seedlings grown in liquid ¼ strength MS medium without charcoal (X0.5)

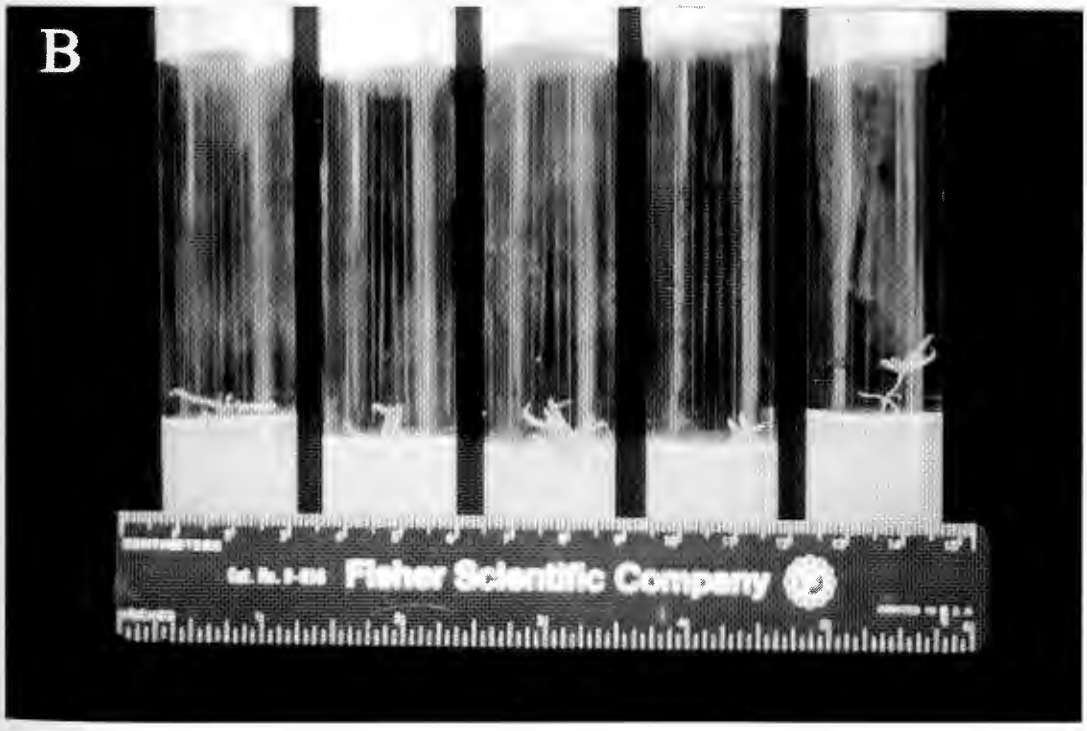

A
B
C
D

Figure 5. *Darlingtonia californica* seedlings - proliferation

A – Separation of clumps of pitchers into clusters with each containing a central rhizome after 6 ½ weeks of growth in liquid medium (Approximately life size)

B – Seedlings grown on ½ strength MS solid medium for 5 months without transfer (X0.7)


A


B
Fisher Scientific Company

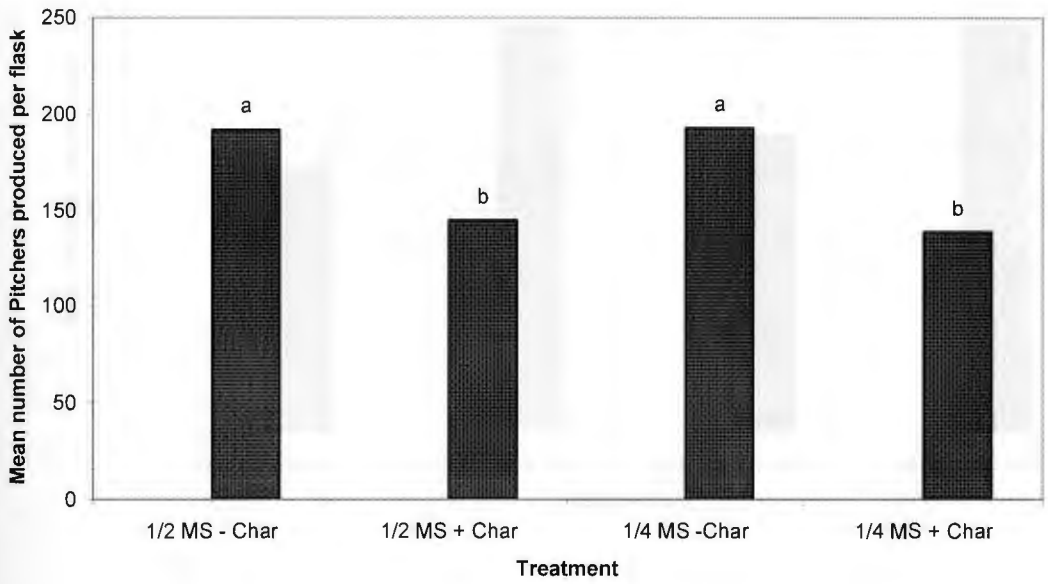


Figure 6. Mean number of pitchers produced per flask per treatment by seedlings of *Darlingtonia californica* in ½ and ¼ strength liquid MS medium with and without charcoal. Incubation period was 6 1/2 weeks. Columns denoted by the same letter were not significantly different. (Fisher's PLSD; P=0.02) Experiments were repeated twice with similar results.

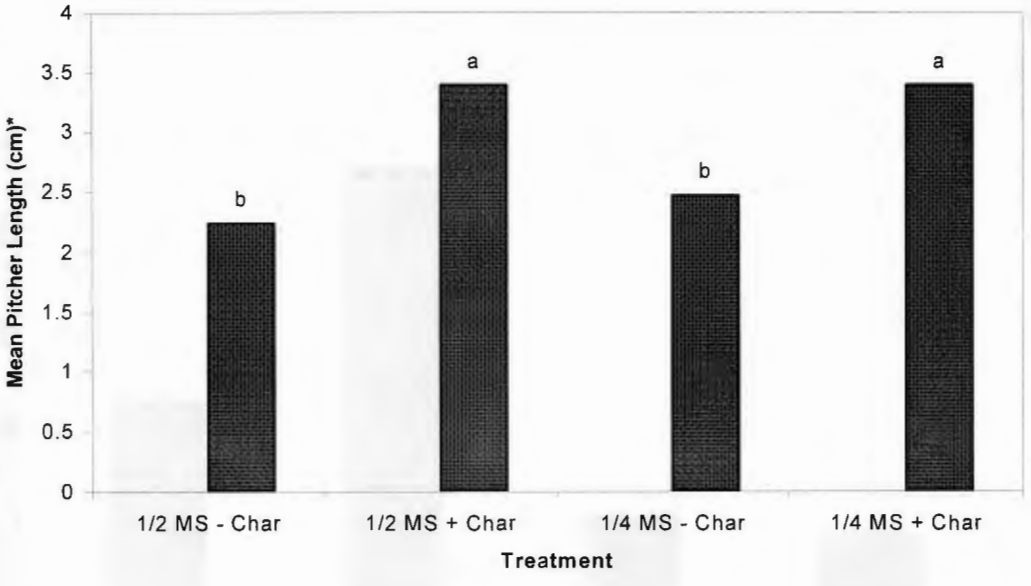


Figure 7. Mean length of pitchers produced per flask per treatment by seedlings of *Darlingtonia californica* in ½ and ¼ strength liquid MS medium with and without charcoal. Incubation period was 6 1/2 weeks. Columns denoted by the same letter were not significantly different. (Fisher's PLSD; P=<0.0001) Experiments were repeated twice with similar results.

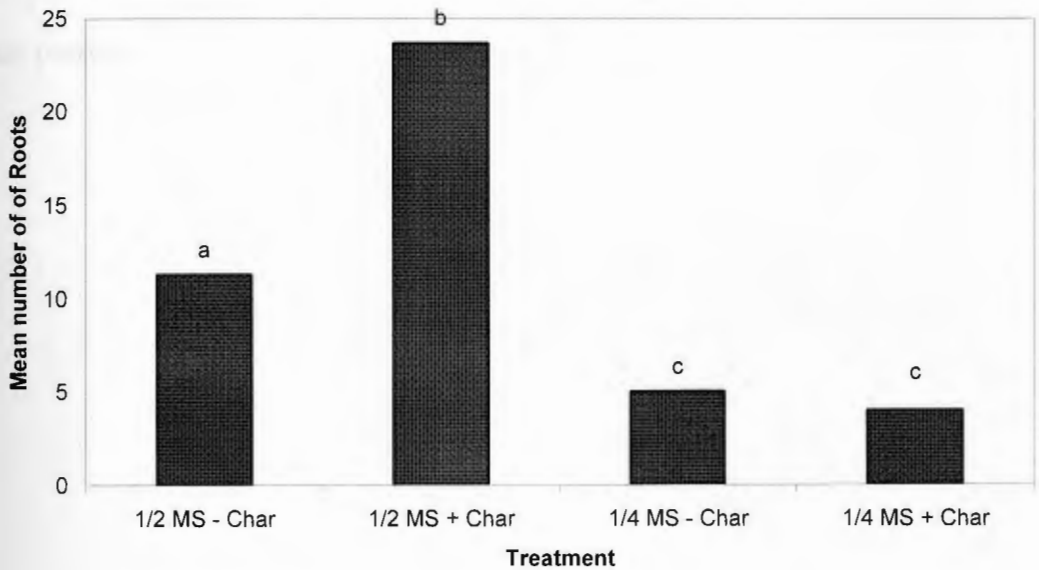


Figure 8. Mean number of roots produced per flask per treatment by seedlings of *Darlingtonia californica* in ½ and ¼ strength liquid MS medium with and without charcoal. Incubation period was 6 1/2 weeks. Columns denoted by the same letter were not significantly different. (Fisher's PLSD; for ab, P=<0.0001; for ac, P=<0.02; for bc, P=<0.0001) Experiments were repeated twice with similar results.

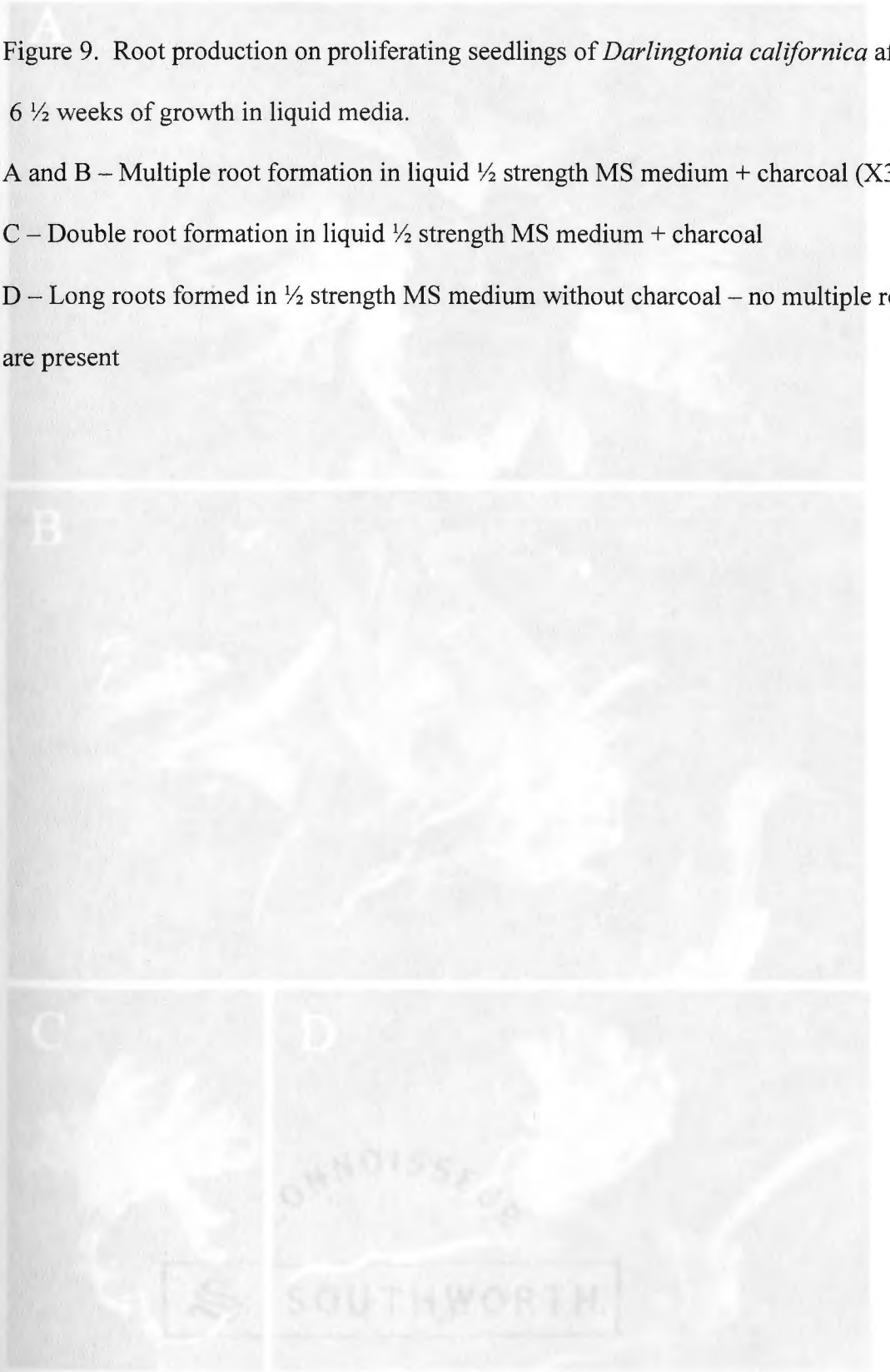


Figure 9. Root production on proliferating seedlings of *Darlingtonia californica* after 6 ½ weeks of growth in liquid media.

A and B – Multiple root formation in liquid ½ strength MS medium + charcoal (X3)

C – Double root formation in liquid ½ strength MS medium + charcoal

D – Long roots formed in ½ strength MS medium without charcoal – no multiple roots are present

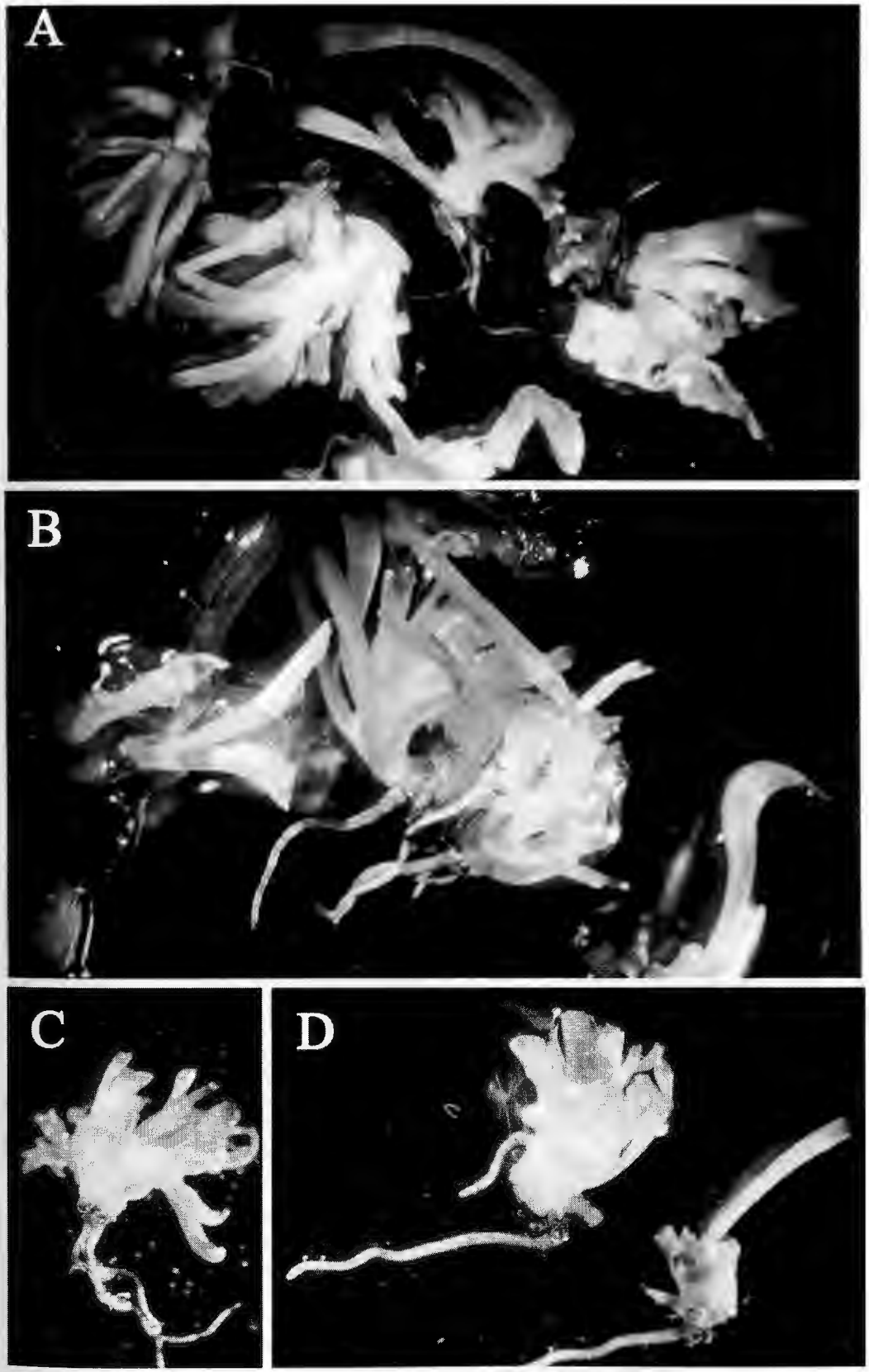
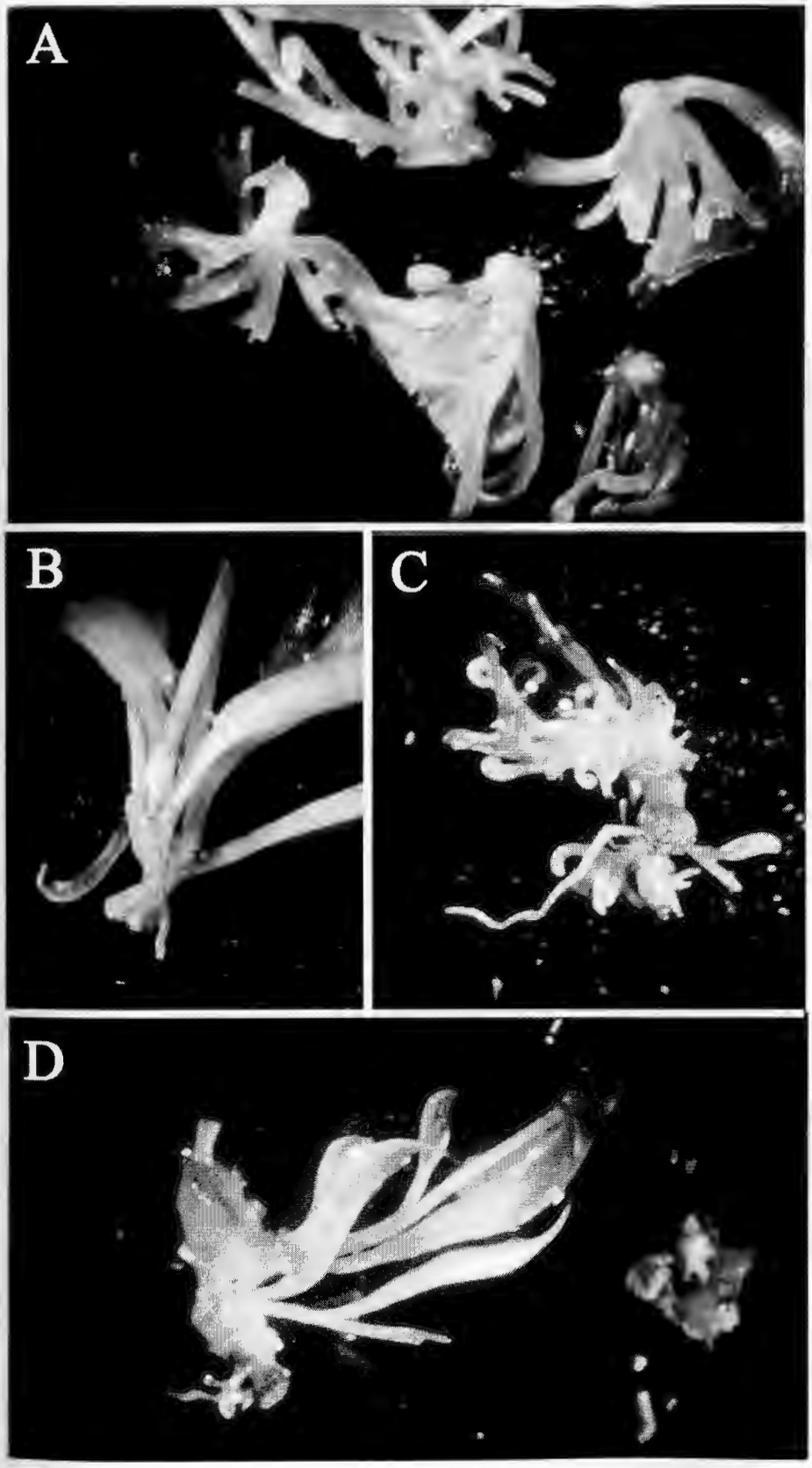

A
B
C
D

Figure 10. Root production of *Darlingtonia californica* after 6 ½ weeks of growth in liquid medium

A – (liquid 1/4 strength MS without charcoal) Several clusters of pitchers showing the absence of roots on the central rhizome (X3)

B and D (liquid 1/4 strength MS without charcoal) Roots when present were usually single in occurrence and short compared to media with charcoal (X3)

C – (liquid 1/4 strength MS with charcoal) Roots generally longer than in ¼ strength without charcoal but still singular in occurrence (X3)


A
B
C
D

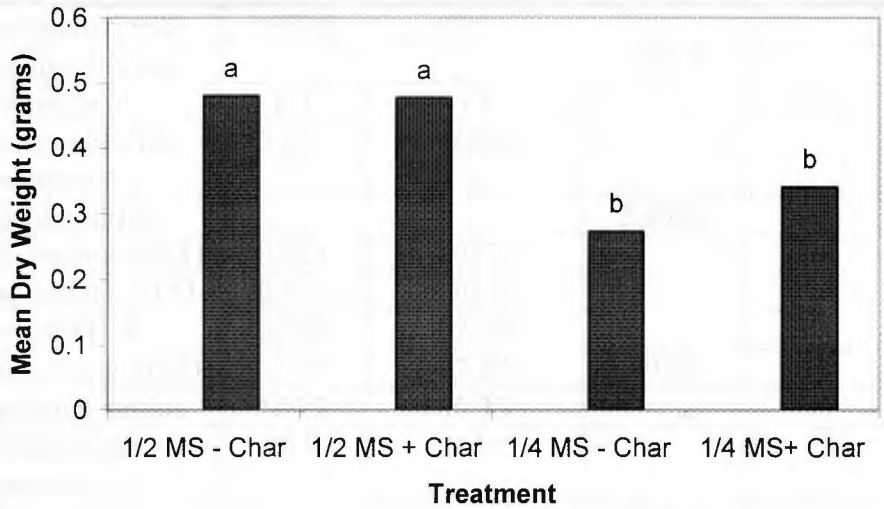


Figure 11. Mean dry weight of plant tissue produced per flask per treatment by seedlings of *Darlingtonia californica* in ½ and ¼ strength liquid MS medium with and without charcoal. Incubation period was 6 1/2 weeks. Each column represents the mean of three replicates. Columns denoted by the same letter were not significantly different. (Fisher's PLSD; $P<0.007$) Experiments were repeated twice with similar results.

Table 1
Comparison of Media Formulations (mg/l)

Medium Components	Full Strength MS Medium	Phytomax Orchid Multiplication Medium	Burgeff's N_3f Medium	Sphagnum-Based Medium
Ammonium nitrate	1650.0	825.0		
Ammonium sulfate			500.0	
Boric acid	6.2	3.1		
Calcium chloride (anhydrous)	332.2	166.0		
Calcium nitrate			2,000.0	
Cobalt chloride·$6H_2O$	0.025	0.0125		
Cupric sulfate·$5H_2O$	0.025	0.0125		
Na_2-EDTA	37.26	37.24		
Ferrous sulfate·$7H_2O$	27.8	27.85	40.0	
Magnesium sulfate	180.7	90.35		
Magnesium sulfate·H_2O	16.9	8.45		
Magnesium sulfate·$4H_2O$			400.0	
Molybdic acid (sodium salt) ·$2H_2O$	0.25	0.125		
Potassium chloride			500.0	
Potassium iodide	0.83	0.415		
Potassium nitrate	1,900.0	950.0		1,000.0
Potassium phosphate (monobasic)	170.0	85.0	500.0	
Zinc sulfate·$7H_2O$	8.6	5.3		
Organics				
Agar	8,000.0	8,000.0	8,000.0	8,000.0
6-Benzylaminopurine (BA)		2.0		
Casein hydrolysate				100.0
Glycine (free base)	2.0		2.0	2.0

Medium Components	MS Medium	Phytomax Orchid Multiplication Medium	Burgeff's N_3f Medium	Sphagnum Based Medium
MES (free acid)		1,000.0		
Myo-inositol	100.0	100.0	100.0	100.0
α-Naphthaleneacetic acid		0.5		
Nicotinic acid (free acid)	0.5	0.5	0.5	0.5
Peptone		2,000.0		
Pyridoxine·HCL	0.5	0.5	0.5	0.5
Chopped live Sphagnum				500 ml beaker, loosely packed
Sucrose	20,000.0	20,000.0	20,000.0	20,000.0
Thiamine·HCl	0.1	1.0	0.1	0.1

Literature Cited

Adamec L (2002) Leaf absorption of mineral nutrients in carnivorous plants stimulates root nutrient uptake. New Phytol., 155: 89-100.

Arditti J (1982) Orchid seed germination and seedling culture – a manual. In: Orchid Biology – Reviews and Perspectives II. (pp. 246-310) Arditti, J. (ed) Comstock Publishing Associates, Cornell University Press. Ithaca, NY

Boulay J (1995) Les plantes carnivores, essais de micropropagation. Bull. Acad. Soc. Lorraines Sci. 34: 151-159

Franck DH (1975) Early histogenesis of the adult leaves of *Darlingtonia californica* (Sarraceniaceae) and its bearing on the nature of epiascidiate foliar appendages. Am. J. Bot. 62: 116-132

Dodds JH & Roberts LW (1995) Experiments in Plant Tissue Culture. Third Edition. (pg. 43) Cambridge University Press. London

George EF & Sherrington PD (1984) Plant Propagation by Tissue Culture. Exergenics Limited. Edington, UK

Hu CY & Wang, P.J. (1983) Meristem, Shoot Tip and Bud Cultures. In: Evans D.A., Sharp W.R., Ammirato P.V. & Yasuyuki Y (eds) Handbook of Plant Cell Culture. (pp.177-227) Macmillan Publishing Co. New York.

Lloyd, E.F. (1942) The Carnivorous Plants. (pp.115-168) Chronica Botanica Co. Waltham, MA.

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497

Redwood G. & Bowling D (1990) Micropropagation of *Nepenthes* species. Bot. Gard. Microprop. News 1: 19-20

Reeves RD, Macfarlane RM & Brooks RR (1983) Accumulation of nickel and zinc by western North American genera containing serpentine-tolerant species. Am. J. Bot. 70: 1297-1303

Schnell DE (2002) Carnivorous Plants of the United States and Canada. Second Edition. (pp. 229-242) Timber Press. Portland, Oregon

Takayama S & Misawa M (1980) Differentiation of *Lilium* bulbscales grown *in vitro*. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation *in vitro*. Physiol. Plant. 48: 121-125

Withner CL (1964) Notes on the cultivation of insectivorous plants *in vitro*. Bull. Torr. Bot. Club 91: 412-413

Ziv M (1979) Transplanting *Gladiolus* plants propagated *in vitro*. Sci. Hort. 11:257-260

MANUSCRIPT III

ESTABLISHMENT OF A MICROPROPAGATION SYSTEM FOR MEMBERS OF THE SARRACENIACEAE

III.

MORPHOLOGICAL RESPONSES DURING *IN VITRO* REGENERATION OF THE NORTH AMERICAN PITCHER PLANTS *DARLINGTONIA CALIFORNICA* TORRY AND *SARRACENIA LEUCOPHYLLA* RAF

ABSTRACT

Darlingtonia californica was regenerated from whole, *in vitro* germinated seedlings and excised segments from *in vitro* generated juvenile pitchers. Whole 2 to 3-week-old seedlings produced protocorm-like bodies and green, leafy callus when cultured on solid Phytomax Orchid Multiplication Medium containing auxin and cytokinin. When divided and subcultured in liquid Phytomax Orchid Multiplication Medium, explants of both protocorm-like bodies and green, leafy callus gave rise to multiple shoots as well as more protocorm-like bodies and green, leafy callus. These could be further divided and subcultured. Transverse segments of excised pitcher leaves from axenically-grown seedlings produced shoots and protocorm-like bodies when subcultured in liquid Phytomax Orchid Multiplication Medium. Unlike *D. californica*, seedlings of *Sarracenia leucophylla* did not readily produce offshoots when incubated on solid media and propagation from a primary rhizome required physical division before subculture to liquid media containing hormones. A protocol for the extraction of intact embryos from selected *Sarracenia* species was developed.

INTRODUCTION

This manuscript is part of a series reporting on the establishment of a micropropagation system for members of the Sarraceniaceae. Manuscripts I and II (this dissertation) describe techniques for the *in vitro* germination, growth and rooting of *D. californica* and the *in vitro* germination of *S. leucophylla*. These previous studies enabled the present investigation of regeneration. This manuscript describes techniques for the *in vitro* multiplication of *D. californica* and *S. leucophylla* with emphasis on the morphogenic potential of whole seedlings and excised pitcher leaves.

D. californica is a North American pitcher plant endemic to the states of Oregon and California (Schnell, 2002). *S. leucophylla* is a southern trumpet pitcher plant distributed along the southeast coast of the U.S. (Cheers, 1992). As with other members of the Sarraceniaceae, these plants have long been objects of interest and study for botanists (see Lloyd, 1942, for early references). Both species possess several attributes that make them suitable for studies in morphogenesis. The shoot systems of *D. californica* and *S. leucophylla* are heteroblastic. In *D. californica* both juvenile and adult pitcher leaves are tubular (epiasidate). However, juvenile leaves lack the hood, keel and fishtail appendage characteristic of the adult leaves (Frank, 1976). Juvenile pitcher leaves of *S. leucophylla* differ from the adult form in that they are narrow, and only gradually widen toward the mouth of the pitcher (Lloyd, 1942). They also lack the abaxial rolled margin present on the mouth of the adult form. Additionally *S. leucophylla* is the only member of the genus *Sarracenia* to form a complete new set of pitcher leaves at the end of the growing season (Schnell, 2002).

Members of the Sarraceniaceae have similar pitcher leaf anatomy. The internal surface of pitcher leaves is divided into several morphologically distinct zones that can be distinguished by epidermal structures. While species and genera vary in the number and size of these zones, the zones function similarly in the attraction, capture and retention of prey and also in the digestion and absorption of nutrients (Givnish, 1989). MacDougal (1903) observed that when *Sarracenia* pitchers were placed in total darkness they doubled in length. He reported that this etiolation was caused by differential changes in zone lengths, with cell numbers increasing in some zones. These early observations indicate that renewed meristematic activity had occurred within some zones. MacDougal's observations are the basis of the pitcher segmentation experiments reported in this manuscript. Additionally, development of epiasidate leaves of the *Darlingtonia* type may be similar to carpel development (Frank, 1975). Carpel margins are formed by the fusion of two epidermal layers that retain considerable meristematic activity following fusion (Walker, 1975). The margins of *D. californica* pitcher leaves undergo a similar fusion (Frank, 1975), which may be associated with retention of meristematic activity.

In contrast to the above morphological similarities, *D. californica* and *S. leucophylla* differ considerably in their growth habit in the natural environment. Seedlings of *D. californica* establish a fibrous root system following germination and during early development of the seedling. Following the production of mature pitchers, the stem becomes plagiotropic, and roots are formed at the nodes (Frank, 1976). It also readily forms offshoots in its natural habitats. On the other hand, *S. leucophylla* reproduces vegetatively much more slowly in its natural habitats (Schnell,

2002). These contrasting growth habits between two species with similar pitcher morphologies offer an opportunity to better understand differences and similarities in morphogenic potential across the two genera. Such an understanding may facilitate micropropagation of members of this and other pitcher plant families such as Nepentheaceae and Cephalotaceae in which epiasidate leaves are produced. Additionally, such studies are particularly timely and important since some members of the Sarraceniaceae have been placed on the endangered species list, e.g., *S. oreophila* and *S. jonesii* (Godt and Hamrick, 1996). To my knowledge, the literature does not contain any reports on *in vitro* morphogenic responses of *D. californica* or S. *leucophylla*.

MATERIALS AND METHODS

Seed Germination

Seeds of *D. californica* were obtained from a commercial supplier and germinated using Methods # 2, # 3, #4 and # 5 as described in manuscript 1 (Table 1) this dissertation.

Callus Culture

Sequential culture of whole seedling on solid and liquid medium

Seeds of *D. californica* were surface disinfested and germinated as described in method # 4 (Table1, Manuscript I). Twelve 3 to 4-week-old seedlings were transferred to each of two, 500 ml Erlenmeyer flasks containing 200 ml of solid Phytomax Orchid Multiplication Medium (POMM, Sigma, St. Louis, Missouri), with 0.5 mg/l NAA and 1.5 mg/l BA. The pH was adjusted to 5.0 prior to autoclaving and before addition of 8.5 g/l agar (Bacto-agar, Difco, Detroit, Michigan, USA). Flasks were incubated in a growth chamber at 27°C ± 2° with 16-hr d^{-1} illumination at 170 μmol m^{-2} sec^{-1}.

Following five weeks of growth, protocorm-like bodies and green, leafy callus were removed from the flasks and subdivided into pieces 3-5 mm in length using a fine pointed scalpel and fine and medium pointed dissecting forceps. Subdivided tissue was distributed evenly among three, 50 ml Erlenmeyer flasks containing 9 ml of liquid POMM (pH 5.0). Culture flasks were incubated in a growth chamber as described above and gently swirled for 15-30 s, every other day. After 5 weeks of growth the tissue from two flasks was photographed *in vitro*, subdivided and distributed evenly among three, 250 ml Erlenmeyer flasks, each containing 75 ml of

fresh medium. The third flask was sacrificed for drawing and observations. Subcultured tissue was photographed after 4 weeks and returned to the growth chamber.

Culture of whole seedlings derived from GA_3 stimulated and unstimulated seeds in liquid medium

Seeds of *D. californica* were germinated with and without GA_3 (Methods # 4 and # 5, Table 1, manuscript 1). Thirty-two, 18-day-old whole seedlings from each treatment were transferred, 4 each, to eight, 25 ml plastic culture flasks each containing 9 ml of liquid POMM and incubated in a growth chamber as described above.

Culture of whole seedlings in full strength liquid MS medium

To assess the effect of full strength MS medium (Murishige and Skoog, 1962) on the growth and development of *D. californica*, 12, 25-day-old seedlings (germinated using Method #4, Table 1, Manuscript 1) were transferred to two, 250 ml Erlenmeyer flasks (6 seedlings per flask) containing 75 ml of liquid MS medium without hormones. Flasks were incubated in a growth chamber (described above) and examined after 28 days of culture.

Pitchers from GA_3-stimulated seeds germinated on solid medium

Seeds of *D. californica* were germinated as described for Method # 5 (Table 1, Manuscript 1), except that seeds were exposed to GA_3 for 12 days instead of 24 h and forty seeds were transferred to each of two, 250 ml Erlenmeyer flasks containing 75 ml of solid POMM (8.5 g/l agar). Following germination, seedlings were allowed to grow for 2 months.

Pitchers from seeds germinated in liquid medium without GA_3

Seeds of *D. californica* were surface disinfested and germinated using Method #1 (Table 1, Manuscript 1) in 50 ml Erlenmeyer flasks and placed on a gyrotary shaker (120 rpm) at room temperature and light conditions. Following 3 months of culture, dense clusters of pitchers were removed and divided. Two clusters (each cluster containing approx. 9-12 pitchers) were transferred to each of three, 500 ml Erlenmeyer flasks containing 50 ml of liquid POMM and placed in the growth chamber.

Organogenesis from whole or fragmented pitchers

Pitchers harvested from seedlings derived from GA_3 stimulated seeds cultured on solid medium were cut into fragments approximately 3-5 mm in length. Six to seven fragments (representing a single pitcher) were transferred to each of three, 50 ml Erlenmeyer flasks containing 10 ml of liquid POMM and placed in the growth chamber. Observations were made over a 45 day period.

Whole pitchers (1-2 cm long) derived from seedlings not exposed to GA_3 and cultured for 45 days in liquid medium were carefully excised using a fine pointed scalpel, fine and medium pointed forceps and a pair of fine pointed iridectomy scissors. Care was taken to insure that rhizomateous tissue was not included at the base of the excised pitchers. In order to ascertain regeneration potential within anatomical pitcher zones, excised pitchers were subjected to the following treatments: 1) whole pitchers were placed (one each) in each of six, 125 mm X 25 mm Pyrex test tubes containing 8 ml of liquid POMM; 2) whole pitchers were cut into 2-3 mm sections and the fragments of 2 pitchers were placed in each of six tubes; 3) whole

pitchers were cut in half and the distal portions placed in one set of six tubes and the basal portions in another set. Additionally, two whole seedlings, 5-months-old and maintained on ½ strength MS medium, were transferred, one each, to each of 2 tubes.

This experiment was repeated one month later with modifications. Pitchers were excised as described above, but pitcher length was 1.0 -1.8 cm. Five treatments were employed (15 test tubes per treatment, each with 10 ml of POMM): 1) whole excised pitchers, one per tube 2) distal half of pitcher, one per tube; 3) basal half of pitcher, one per tube; 4) distal half fragmented (3-4 fragments), one set per tube; 5) basal half fragmented (3-4 fragments), one set per tube. Additionally, pitchers approximately 1.8 cm in height were cut into nine fragments with the center fragment excised first (fragment #5) and placed one each, in numbered tubes. This was replicated 4 times. The duration of this experiment was 30 days.

Multiplication of *S. leucophylla in vitro*

Two, 2-week-old seedlings of *S. leucophylla* that had germinated on MYP (from surface disinfestations experiments Manuscript 1, this dissertation) were transferred to 10 ml of ½ strength MS medium + vitamins in 125 mm x 25 mm test tubes and placed in a growth chamber. Temperature and light conditions were as described in Manuscript 1, this dissertation. After 30 days they were transferred to solid POMM. After two months of growth they were subcultured to a 500 ml Erlenmeyer flask containing 50 ml of liquid POMM. After 105 more days the remaining tissue was divided into several pieces, each containing a portion of the rhizome and several pitchers, and distributed among three, 250 ml Erlenmeyer flasks, each containing 75 ml of liquid POMM. Four pitchers were excised from rhizomes

and cut into seven fragments each and placed randomly in 4, 25 ml plastic culture vessels each containing 9 ml of liquid POMM. Following another 45 days of culture the growth in the flasks was documented photographically.

Extraction of intact endosperm and embryos

Seeds of S. *leucophylla* and *S. purpurea* were treated in concentrated H_2SO_4 for 10 min followed by three, 5 min rinses in sterile distilled H_2O and then dissected and the embryos removed. Dissections and extractions were performed with the aid of a dissecting microscope, fine pointed forceps, and micro-dissection needles. A fine pointed hypodermic needle attached to the blunt end of a dental pick served as a micro-scalpel. Seed coats were split, by making a single, incision along the long axis of the seed. Portions of the micropylar and chalazal end of the seed were removed with the micro-scalpel and the coat gently teased away from the endosperm using forceps and micro-dissection needles. After removal of the seed coat, an oblique cut was made across the micropylar region of the endosperm. Embryos were teased out by manipulation and gentle pressure on the chalazal end and/or by carefully removing layers of endosperm tissue using the edge of a micro-scalpel as a micro- scraper. Alternatively, following treatment in acid, seeds were placed in 3 ml of sterile H_2O in 1.5 ml plastic centrifuge tubes and spun on a vortex mixer for 3 min. followed by a change of H_2O and gentle agitation with the tip of a sterile Pasteur pipet. This process was repeated several times until most or all of the seed coats were removed. Residual coat material was removed under the dissecting microscope by gentle teasing using micro-dissection instruments. Embryos were extracted as described above. These

procedures were performed on the lab bench or, in order to obtain sterile material for experiment, in a laminar flow hood.

RESULTS

Production of protocorms and callus from tissue derived from whole seedlings by sequential culture on solid and liquid medium

When 4-week-old seedlings were placed with their longitudinal axis flush against the surface of POMM (Fig.1A), seven of 12 began to brown during the first week of culture. These seedlings were completely brown by the end of the second week and produced no new growth for the duration of the experiment. Five seedlings remained green with some yellowing of the hypocotyls and cotyledons (3 in flask 1, 2 in flask 2). Their cotyledonary nodes began to green and swell during the second week, forming hard green callus and/or green leafy callus (GLC) by the end of week 3. These growths eventually obscured the cotyledonary nodes and also appeared along the hypocotyls proximal to the node. GLC also arose basipetally toward the root tip. No increase in length of the cotyledons, hypocotyls, or primary root was observed. After 70 days of culture two hard callus masses resembling orchid protocorms (protocorm-like bodies, PLB) had formed (Fig.1B). The GLC remained leafy (Fig. 1C) and many small, spherical, bud-like structures appeared on the surface of the tissue. These structures formed on both types of callus and were morphologically similar to developing orchid protocorms (Fig.1D). Similar callus forms in leaf cultures of *Cattleya* orchids (Fig.1E). When removed for subculture (first subculture) the GLC was more friable than the PLB. After approximately 15 days of subculture, both PLB and GLC began to form new shoots, GLC and spherical bud-like structures, which later formed PLB. PLB could sometimes be separated from parent tissue by gently swirling the medium in the flask, and larger PLB sometimes separated

spontaneously in culture. These large PLB resembled mature orchid protocorms (compare Fig.2Aand 2B). After 44 days of subculture, dense clumps of pitchers and callus were produced (Figs. 2C and 2D). The cultures appeared healthy and new growth initiated from the circumference of the collective tissue mass.

When the masses were divided for subculture (second subculture) numerous plantlets lacking roots, large and small PLB, and various amounts of GLC in several stages of development were observed. Tissue masses were divided into several large clusters, but the original explant tissue could not be identified. Yellowing and browning of some tissue during the first week of the second subculture was followed by a 1 to 1 ½ -week quiescent period before new growth was observed. A color change from yellow-green to green was the first indication that growth had resumed. After 45 days of growth the flasks were photographed (Fig.3And B). Shoots generated from subcultures of PLB and GLC were morphologically similar to shoots produced from whole seedlings (Fig. 3C). As of this writing the flasks are in a growth chamber and have produced a large amount of tissue for further subculture and experimental use.

Production of callus from whole seedlings derived from GA_3 stimulated and unstimulated seeds and cultured in liquid medium

After 36 days of subculture five seedlings in the treatment without GA_3 had swollen nodes and leaf bases and had produced new pitchers (Fig.4A, compare with Fig. 1A). PLB and GLC were present on only 1 of these seedlings. The others had completely browned by the end of the experiment. In contrast, eight of 16 seedlings from seeds stimulated by GA_3 showed new green growth and on 3 of these PLB and

GLC could be observed (Fig. 4B). The remaining 8 seedlings either browned or yellowed by the time the experiment was terminated and produced neither PLB nor GLC.

Culture of whole seedlings on full strength liquid MS medium –

When 12, 25-day-old seedlings were cultured in full strength MS medium, no evidence of multiplication or morphogenesis was observed after 30 days (Fig. 4C). One seedling browned, and 11 remained green with some yellowing. Some browning of pitchers was observed.

Organogenesis from whole and fragmented pitchers

When fragments of pitchers harvested from plants grown from GA_3 stimulated seeds on solid medium were cultured in liquid POMM, new growth was observed in 2 of 3 flasks after 45 days (Fig. 5A). One fragment of 7 in flask #2 has formed 5 well-developed pitcher leaves (Fig. 5B) and several small PLB were clustered around the center of leaf origin (Fig. 5B). These small PLB could be separated from the parent tissue (Fig. 5C). In flask # 3, two of seven fragments had produced two PLB (Fig. 4D and E). One PLB had given rise to a cluster of small pitchers (Fig. 4D). In this experiment three of 20 pitcher fragments produced new growth. All six pitcher fragments in flask #1 gradually browned and died.

The pitchers used as source material for subsequent experiments were plantlets derived from non-GA_3 treated seeds and subcultured in liquid POMM for 45 days. During this time the plantlets multiplied and completely covered the bottom of the flask (Fig. 6A). A few small roots originating from central cluster rhizomes were

visible with the aid of a hand-lens. The plantlets appeared chlorotic at the time pitchers were excised and fragmented.

In four of the six tubes in which pitcher fragments were cultured, new growth occurred.(Fig. 6B). Eleven of a total of 50 fragments produced new growth, and the rest slowly browned. In tubes one and two, 4 fragments produced new growth, tube three contained 10 fragments with two producing new growth, all fragments browned in tubes four and five, and one fragment in tube six produced new growth. The fragments varied from small spherical bodies (Fig. 6B, inset), small PLB, to large hard, dark green callus (Fig. 6B). PLB closely resembled orchid protocorms (Fig. 6C, compare to Fig.1D). Whole pitchers usually turned brown, usually starting from the basal end. Most had completely browned after 3 weeks of culture. Both distal and basal halves of pitchers slowly browned and died with the basal halves browning more quickly than the distal halves. The distal tip of whole pitchers and distal halves sometimes remained green and became somewhat translucent and yellow before finally browning. Whole seedlings ultimately turned brown. However, one started to show new growth at the end of the experiment. This growth appeared to be from pitcher elongation and was accompanied by dark green coloration. Adventive structures were not observed.

The variable results noted above indicate that morphogenic potential of pitcher fragments may depend on the zone of the pitcher from which fragments were derived, so fragments were cultured individually and their position within the pitcher was noted when the experiment was repeated. However, very little regeneration was obtained in this experiment. One of 15 whole pitchers showed new growth from the tip (Fig. 7A)

after 30 days of subculture. One of 15 basal halves of bisected pitchers formed a PLB and leaf-like structures at the distal end. This growth lost its coloration in the last 10 days of the experiment (Fig. 7B). No growth was observed in any other explants. Whole pitchers usually browned first from their basal ends and after one week of culture, the distal ends appeared larger and more translucent and were the last area to brown. Fragmented pitcher halves were paired so that fragments of the same pitcher were in adjacent tubes. Although all ultimately turned brown, the process of browning commenced at different times during the experiment and appeared synchronized between complementary tubes containing fragments of the same pitcher. The same was true for serially sequenced fragments. All fragments from the same pitcher began to brown at approximately the same time. Browning first appeared in the basal fragments in all treatments.

Multiplication of *Sarracenia leucophylla in vitro*

Seedlings of *S. leucophylla* grew slowly on solid ½ strength MS medium. After two months of growth they had produced few pitchers and showed little tendency toward adventive multiplication. Transfer to solid POMM did not appear to have much effect. Although they did produce more pitchers and rhizome length appeared to increase slightly. Following transfer to liquid POMM, clusters of pitchers were formed. Pitcher clusters could be divided by rhizome cutting and subculture of clusters resulted in rapid multiplication, continued increase in pitcher height, and developmental movement toward adult pitcher morphology (Fig. 8A, B, and C). Excised pitcher fragments gradually browned and died. No new growth was observed on any of the subcultured pitcher fragments after 45 days of subculture.

DISCUSSION

Conditions for the production of protocorm-like bodies and callus

Because whole, axenically germinated seedlings have been used to initiate clonal multiplication through direct differentiation of shoots in several micropropagation systems (Malik and Saxena, 1992; Teo et al., 2001; Parliman et al., 1982a), the formation of callus and PLB from whole, *in vitro* germinated seedlings of *D. californica* was unexpected. Additionally, Rathore et al. (1991) reported the recalcitrance of whole, *in vitro* generated seedlings of the rare pitcher plant *Nepenthes khasiana* to produce callus when exposed to phytohormones in culture. In contrast, during this study, *D. californica* readily produced several types of callus and regenerative tissue distinguishable by differing gross morphology. During the last three decades of tissue culture, hypocotyls and cotyledons have been excised from axenically germinated seedlings of a variety of plant species and subcultured to initiate callus and somatic embryogenesis (see Kohlenbeck, 1978, for early review of somatic embryogenesis).

Similar developmental phenomena also were observed by Truscott (1966) in his studies of morphogenesis in *Cuscuta gronovii*. He observed that original explants (extracted, intact embryos) showed little development but gave rise to many adventive buds. Callus-like outgrowths formed at the bases of adventive buds and eventually formed many smaller buds. These phenomena are similar to that observed in whole cultured seedlings of *D. californica*. Additionally, Parliman et al., (1982b) described similar structures arising from cultured leaf tissue of *D. muscipula*. These structures were considered to be adventitious. Also, they report that in mature cultures, only the

first bud appears to be adventitious and the subsequent buds appear to be lateral buds derived in sequence from it. This could also be the case in *D. californica* since preformed buds most likely do not occur on the hypocotyls and cotyledons.

Although histological studies were not performed during this study, the nature of PLB and GLC can be interpreted based on the anatomy and growth habit of the shoot system of *D. californica* seedlings. During leaf development in *D. californica* the newest emerging leaf forms a clasping base that nearly encircles the leaf primordium of the next developing pitcher leaf (Frank, 1976). This imbricate developmental pattern places axillary buds in close proximity prior to internode elongation. If these buds are activated by the presence of phytohormones they may give rise to the structures described in this manuscript as PLB. These structures would appear clustered at shoot tips. Alternatively, PLBs produced along the hypocotyls and cotyledons may be adventitious buds (Goebel's "Anlagen" – see Appendix C).

The ability of PLB and GLC to proliferate when divided and subcultured was not surprising since, at the time of division, considerable cytodifferentiation and morphogenesis had occurred. When transferred to fresh medium, the rapid formation of many shoots in dense clusters may have been the result of simultaneous activation of the PLB produced during the initial culture period. This rapid formation of shoots in dense clusters supplies evidence that PLB are bud-like in behavior.

The initial yellowing and dieback of some pitchers after subculture may have been associated with medium pH. Although pH was not monitored during culture, a change from the original pH of 5.0 may have occurred. Liquid cultures of the Australian pitcher plant *Cephalotus follicularis* were observed to die back when

transferred to fresh medium (Adams et al., 1979). It was suggested that this phenomenon was pH dependent because it occurred when medium at pH 5.7 was used for subculture.

In this study lack of rooting may have been due to high auxin levels. The auxin in the medium coupled with the natural production of auxin by proliferating shoots may have led to levels inhibitory to root development. High auxin levels have been shown to cause callus formation at the shoot base of *in vitro* cultured plants and inhibit root formation (Lane, 1979).

Effect of GA_3 stimulation on whole seedlings cultured in liquid medium

The lack of response of seedlings, without prior GA_3 stimulation, to incubation in liquid medium with phytohormones is puzzling. Protocorm-like bodies and green, leafy callus were produced by non-stimulated seedlings cultured on the same medium in solid form. Seedlings transferred to solid medium were 21-28 days old vs. 15-18 days old for the seedlings transferred to liquid medium. It has been established that response in culture varies considerably in some species depending on physiological age, endogenous hormone concentrations as well as the history of the explant material (Hu and Wang, 1982).

The death of seedlings incubated in full strength MS medium was not unexpected. In nature, carnivorous plants generally inhabit nutrient poor habitats (Adamec, 1997) and media containing reduced salt concentrations have been successfully employed for *in vitro* culture of these plants.

Regenerative potential of fragmented pitchers

This series of experiments tested the hypothesis that regenerative potential varies throughout specific zones of pitcher leaves of the Sarraceniaceae. This hypothesis was based on earlier observations on elongation potential of etiolated pitcher leaves of *Sarracenia* (MacDougal, 1903), the mode of leaf histogenesis (Frank, 1975,1976) and carpel development (Walker,1975). Walker describes placental tissue as arising from the fusion of two carpel epidermal layers which redifferentiate as placental tissue with great morphogenic potential (production of ovules). Fusion of epidermal layers also occurs during leaf histogenesis in the Sarraceniaceae, ultimately resulting in the familiar wing of *Sarracenia purpurea* and the keel of *Darlingtonia californica*. In other species of Sarraceniaceae this fusion line has been termed a suture (Lloyd 1942).

The adaxial surface of the pitcher leaf (the boundary between the interior of the pitcher and the external environment) is a highly differentiated epidermis occurring in discrete zonation. Each zone is characterized by specific epidermal structures such as downward pointing hairs, smooth waxy surfaces and, in some cases, digestive or absorptive glands. These zones function to direct the prey into the interior of the pitcher and in digestion and absorption of nutrients. The suture line, or wing, formed by the fusion and subsequent transdifferentiation of epidermal tissue is vascularized and continuous through all zones from the base of the pitcher to its tip (see Lloyd 1942, pp.19-36). Because pitcher development was continuous in *vitro*, it was hypothesized that pitchers fragmented to approximate the natural zonation might

retain regenerative or developmental potential either at zone boundaries or along the suture line.

The first experiment was performed to quickly assay the ability of randomly fragmented pitchers to produce new growth from the excised fragments. The results of this experiment supported the hypothesis with new growth in the form of PLB, pitcher leaves and one whole plantlet being produced from several of the fragments.

In a second experiment, fragmented whole pitchers produced PLB, small green spherical bodies, and large, dark green callus. Why the cultured whole, distal, and basal halves of pitchers ultimately browned and died is not understood. One possibility is, the fragmented pitchers may be stimulated by a collective wound response to produce callus because of the number of wounded fragments placed together in the same test tube. This response may not have occurred in the tubes containing whole or half pitchers because of a low level of signal molecules since less wound surface area was exposed to the medium. Another possibility is that the tissue zone or zones capable of the response were located interior to the cut and may require being cut in order to initiate the response. These hypotheses could not be investigated because all the fragments from one pitcher were placed collectively in individual test tubes.

Although the results of the last experiment in this series were inconclusive some useful information was gathered. For instance, whole pitchers usually browned from the basal end first with the browning proceeding acropetally. The distal tips were also observed to become enlarged and translucent before ultimately browning. The only growth to appear from whole pitchers was from the distal end. These three

observations, though based on a small sample size, suggest that a closer look at the distal zone may be the first step in planning future investigations. Additionally, because regenerative structures were produced by tissue with prior exposure to GA_3 and tissue without GA_3 exposure, the role of this hormone in the induction of renewed meristematic activity, as observed in these experiments, still remains unclear.

Multiplication of *Sarracenia leucophylla in vitro*

The slow growth of *S. leucophylla* on solid medium without hormones may be related to its natural habit. In nature this species does not proliferate by extensive secondary rhizome formation as does *D. californica* (Schnell, 2002). Instead, it produces new shoots from the primary rhizome. Commercial propagation is accomplished through cuttings taken from the primary rhizome that include at least one root (Pietropaolo and Pietropaolo, 1986, D'Amato, 1998). This form of propagation is very slow and faster rates may be obtainable through *in vitro* culture. Additionally, cuttings must be taken them just before the new growing season. This restriction does not apply to *in vitro* culture.

The lack of response of *S. leucophylla* when transferred to solid POMM containing auxin and cytokinin may have been caused by its previous long period of culture on solid medium. Prolonged culture with depletion of nutrients and leaching of toxic metabolites from the plants into the medium may have caused arrested growth. This hypothesis is supported by its rapid proliferation and growth when transferred to liquid POMM. Serial subculturing can break the recalcitrant state of many species as well as transfer to a liquid shaking system (Hu and Wang, 1984).

Renewed initiation of growth and multiplication of *S. leucophylla* occurred when transferred to liquid medium without shaking.

Failure of pitcher fragments of *S. leucophylla* to form new growth in liquid culture could be due to one of several factors including developmental age, an inherent inability to form new centers of meristematic activity, or the sample size may have been too small.

Extraction of intact endosperm and embryos

The removal of seed coats using the vortex mixer method was preferable over the first method outlined. It allowed more seeds to be processed in a shorter time and extensive manipulation of seeds to completely remove the coat was usually not necessary. With practice, success rates reached seven out of 10 attempts and often the endosperm could be left intact with the exception of a small incision at the micropylar pole. This occurred when gentle pressure at the chalazal pole forced the embryo to slide out through the incision. Success was dependent upon the position of the embryo within the endosperm.

Summary

Darlingtonia californica can be multiplied either through serial subculture of aseptically germinated seedlings or from fragmented juvenile pitchers in medium containing phytohormones. A variety of morphogenic responses are rapidly inducible with a commercial medium (POMM). This suggests *D. californica* as a suitable research organism for continued study of regeneration events and for optimization of a system for micropropagation.

Sarracenia leucophylla is promising as a subject for micropropagation but its use as a research organism may be limited until techniques to initiate *in vitro* responses are developed. However, the establishment of a protocol for the surgical extraction of intact endosperm and embryos of *Sarracenia* species can provide material to test the suitability of these species for endosperm and embryo culture experiments.

This study has achieved the initial steps in the stimulation of morphogenic responses in *D. californica* and has brought *S. leucophylla* into the study arena. Additional concerns are success in *ex vitro* acclimatization of plantlets and effects of long-term culture on genetic stability.

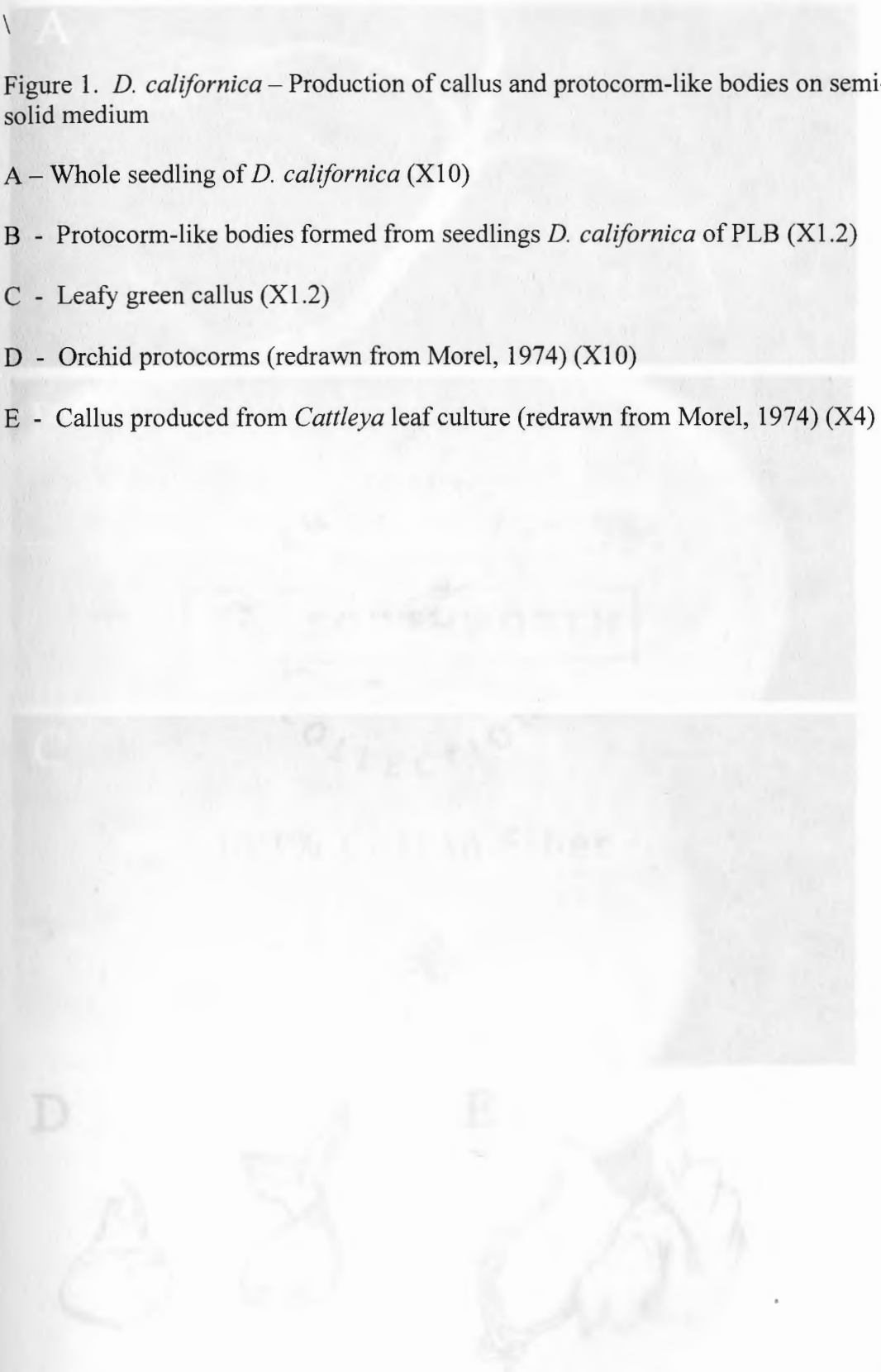


Figure 1. *D. californica* – Production of callus and protocorm-like bodies on semi-solid medium

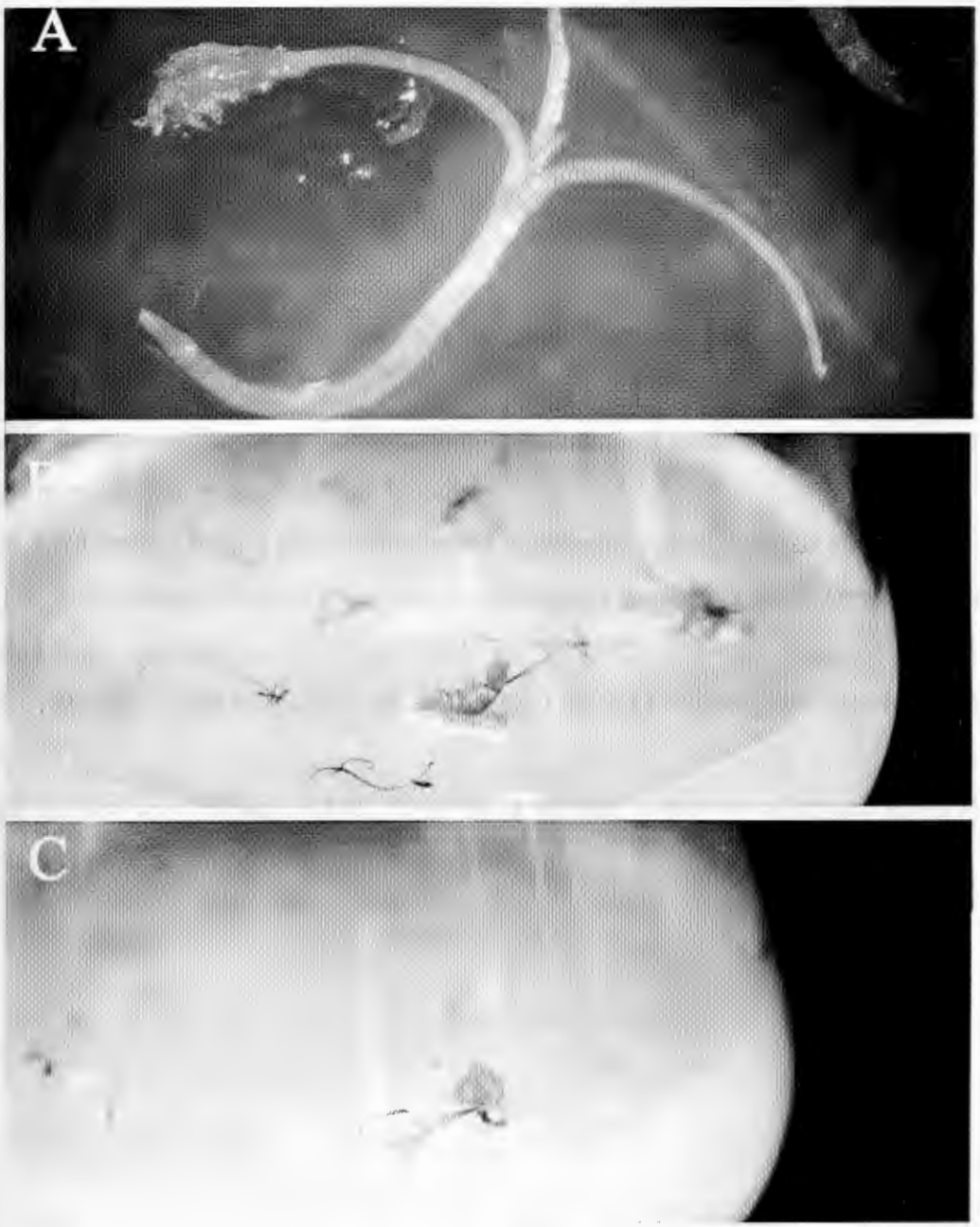
A – Whole seedling of *D. californica* (X10)

B - Protocorm-like bodies formed from seedlings *D. californica* of PLB (X1.2)

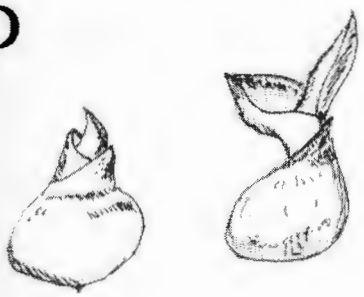
C - Leafy green callus (X1.2)

D - Orchid protocorms (redrawn from Morel, 1974) (X10)

E - Callus produced from *Cattleya* leaf culture (redrawn from Morel, 1974) (X4)



D



E

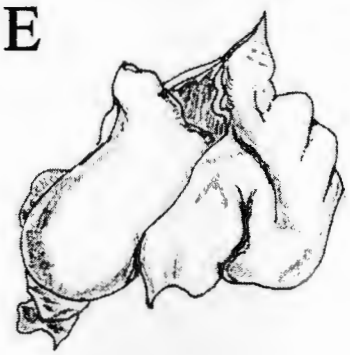


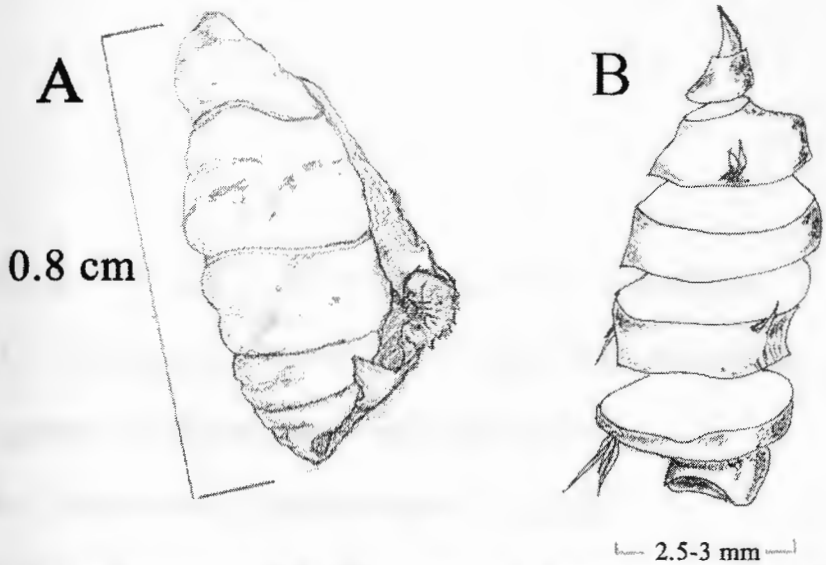
Figure 2. *D. californica* – Comparison of protocorm-like bodies with orchid protocorms – proliferating masses of pitchers and tissue

A - Protocorm-like body that separated spontaneously from parent tissue during liquid culture (drawn by Leslie Anne Uhnak).

B - A Cattleya orchid protocorm similar in morphology to the PLB shown in Fig. 1A. It has been cut for *in vitro* culture in the same manner as PLB were prepared in this study (redrawn from Morel, 1974).

C - Dense clumps of pitchers and callus after 44 days of liquid culture in POMM (X0.7)

D - Close-up of a flask in Fig. 2C showing various tissue responses and new growth new growth at the edge of the tissue mass


A
0.8 cm
B
2.5-3 mm


C


D

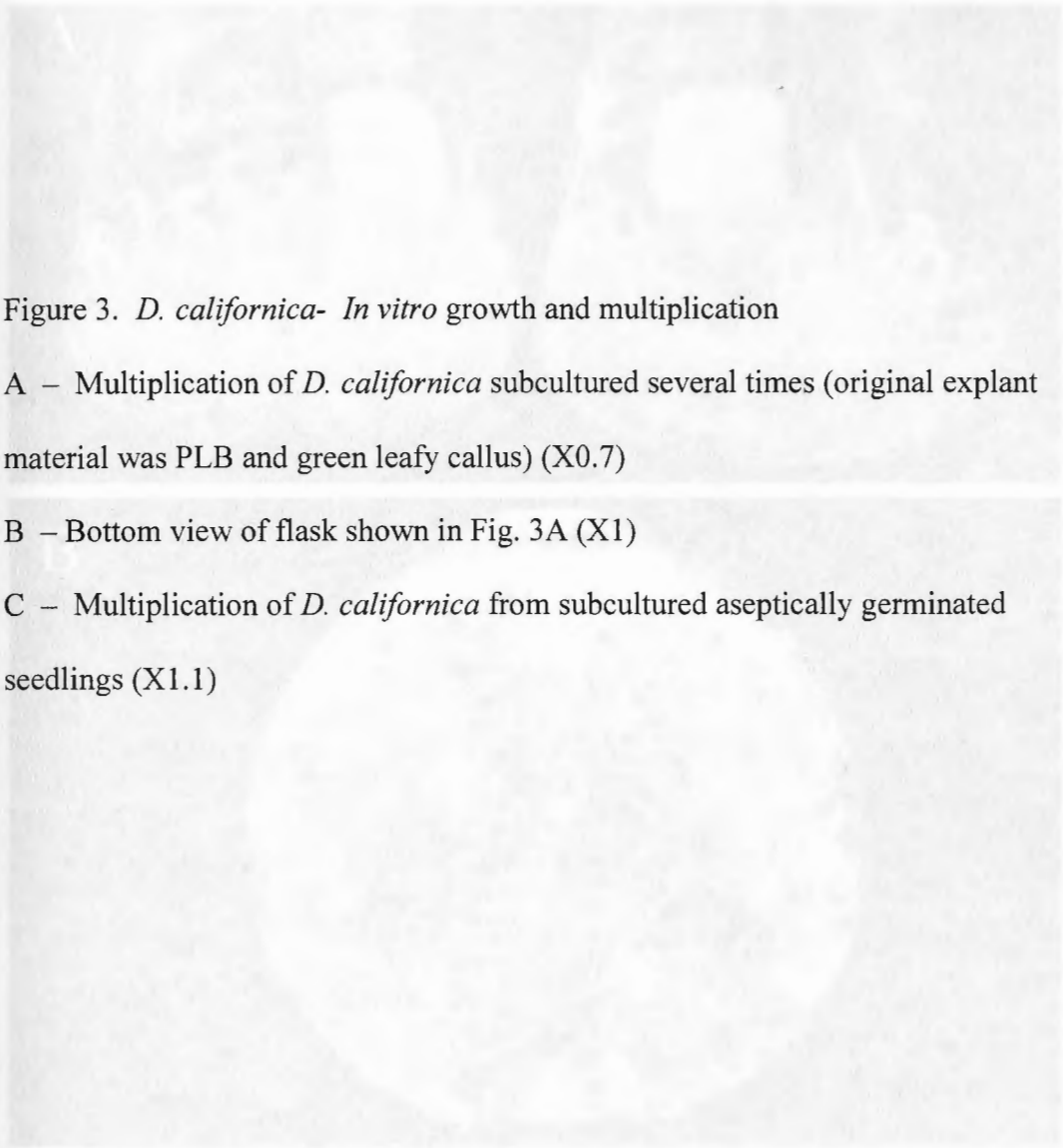
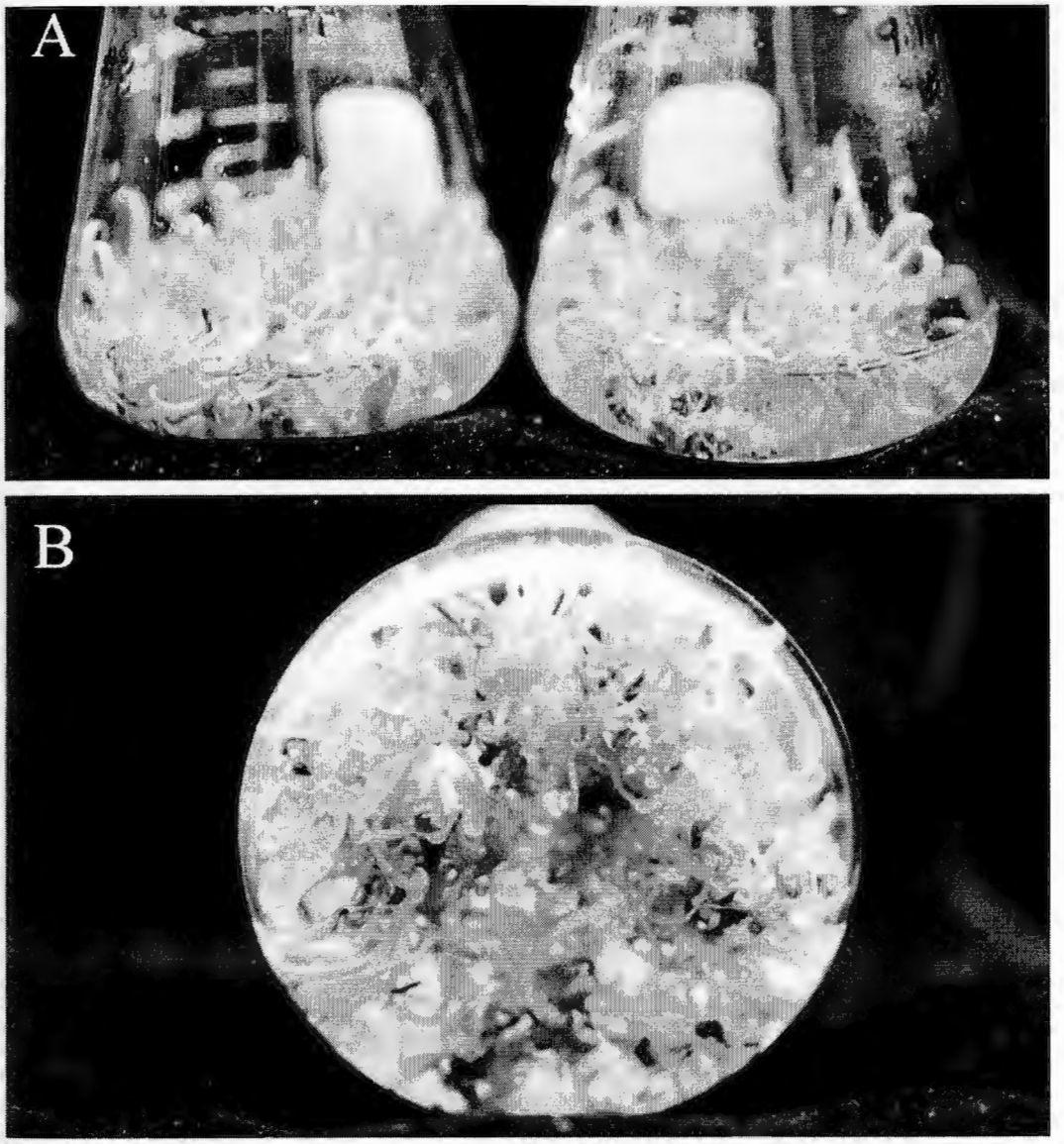


Figure 3. *D. californica- In vitro* growth and multiplication

A – Multiplication of *D. californica* subcultured several times (original explant material was PLB and green leafy callus) (X0.7)

B – Bottom view of flask shown in Fig. 3A (X1)

C – Multiplication of *D. californica* from subcultured aseptically germinated seedlings (X1.1)


A
B
C

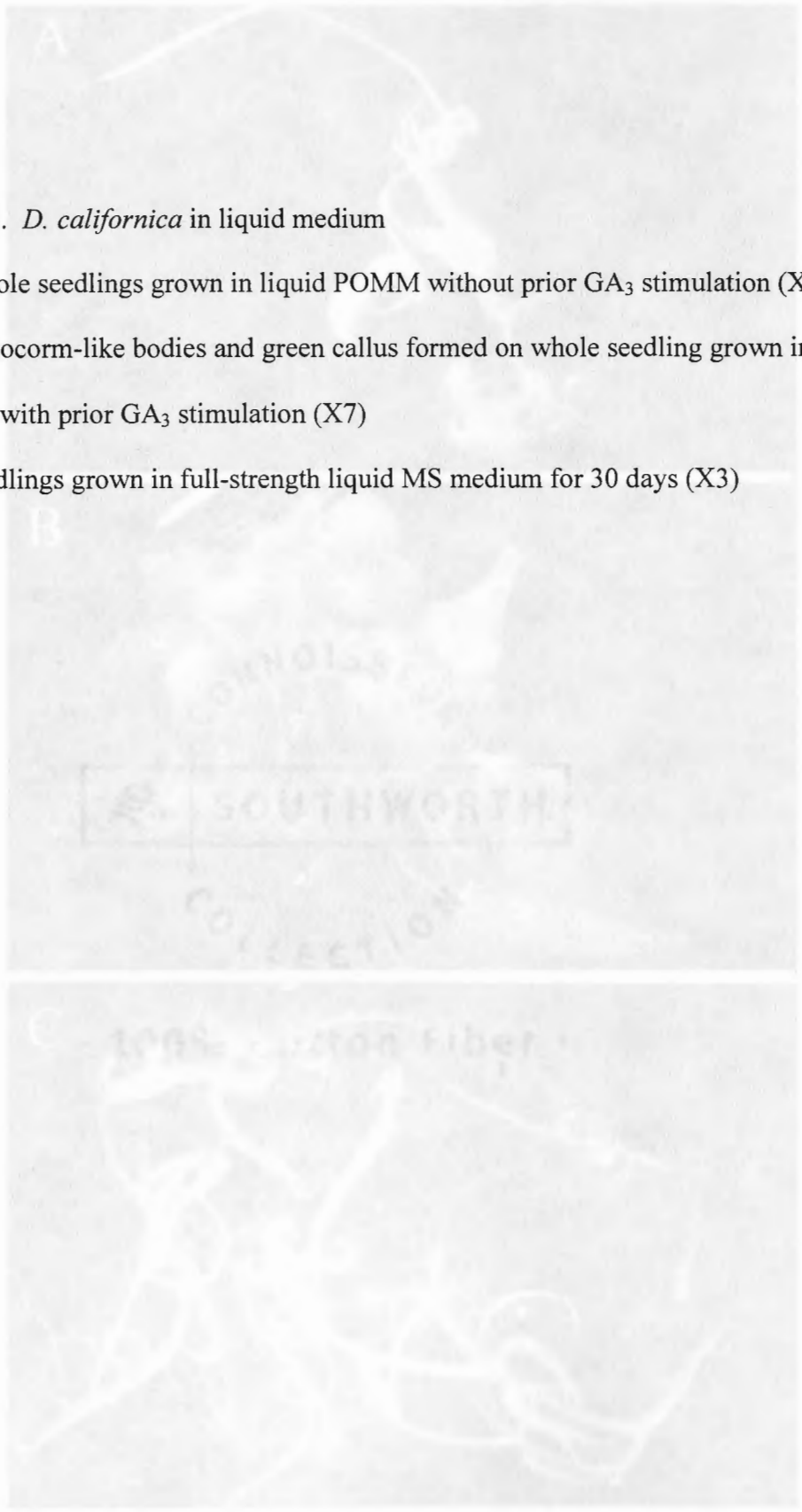


Figure 4. *D. californica* in liquid medium

A – Whole seedlings grown in liquid POMM without prior GA_3 stimulation (X3.5)

B – Protocorm-like bodies and green callus formed on whole seedling grown in liquid POMM with prior GA_3 stimulation (X7)

C – Seedlings grown in full-strength liquid MS medium for 30 days (X3)

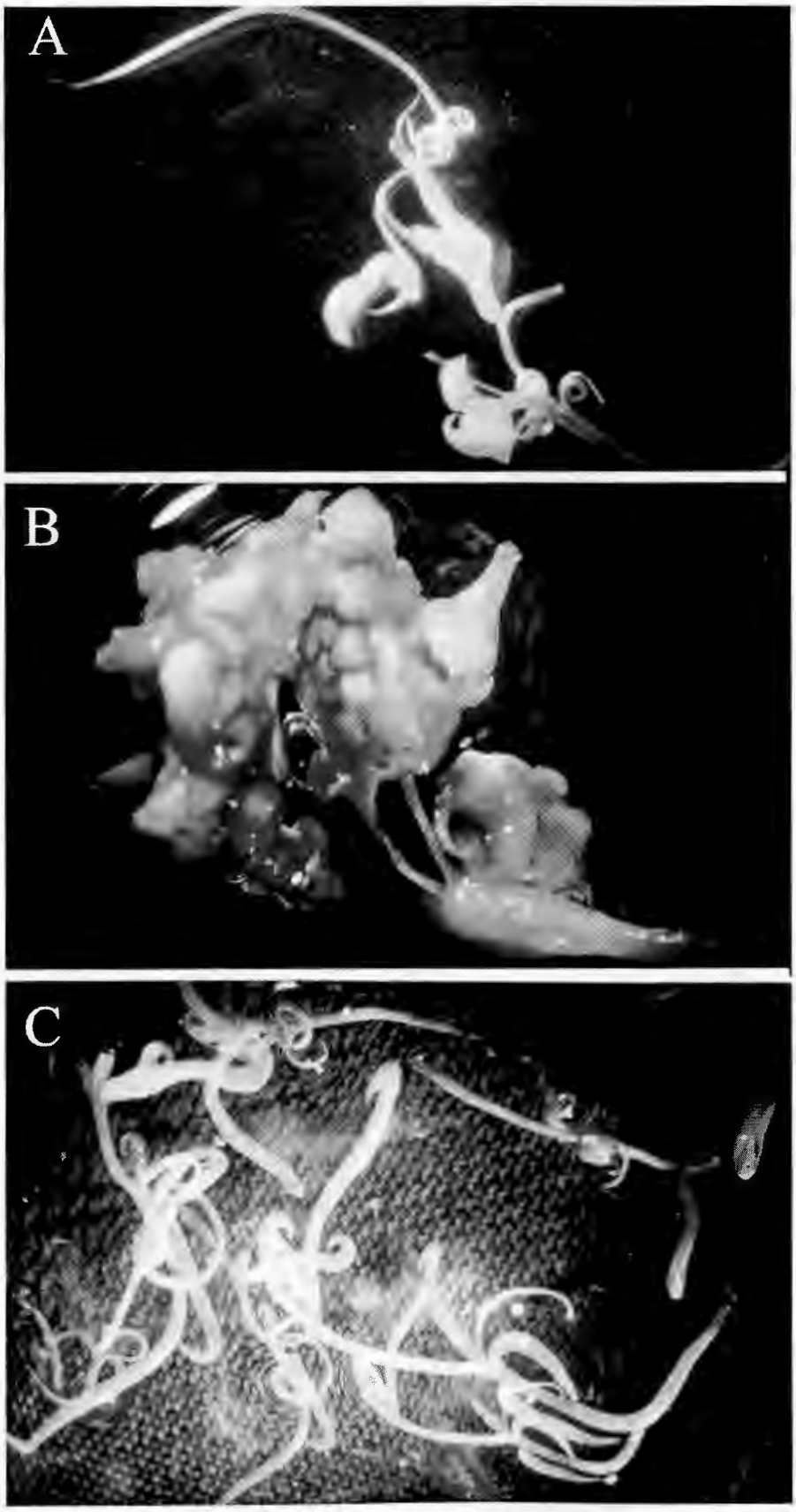

A
B
C

Figure 5. *D. californica*- Morphogenic structures produced from fragmented pitchers.

A - Flasks with shoots and protocorm-like bodies (X1.1)

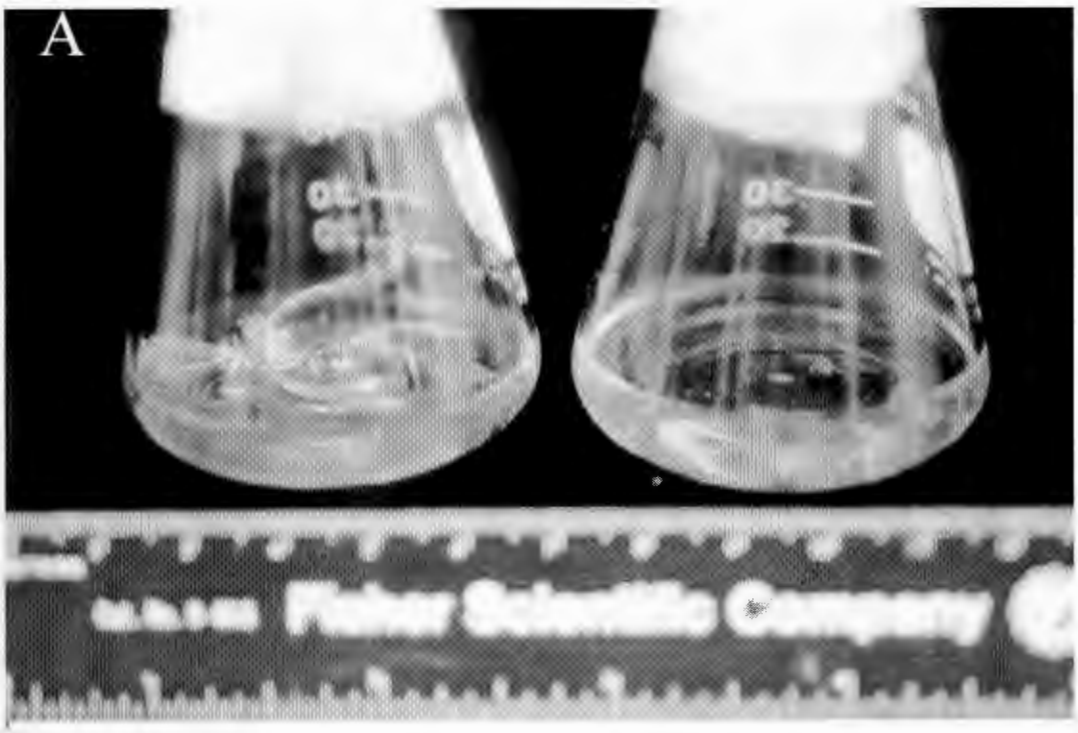
B - Well-developed plantlet formed from pitcher fragment.

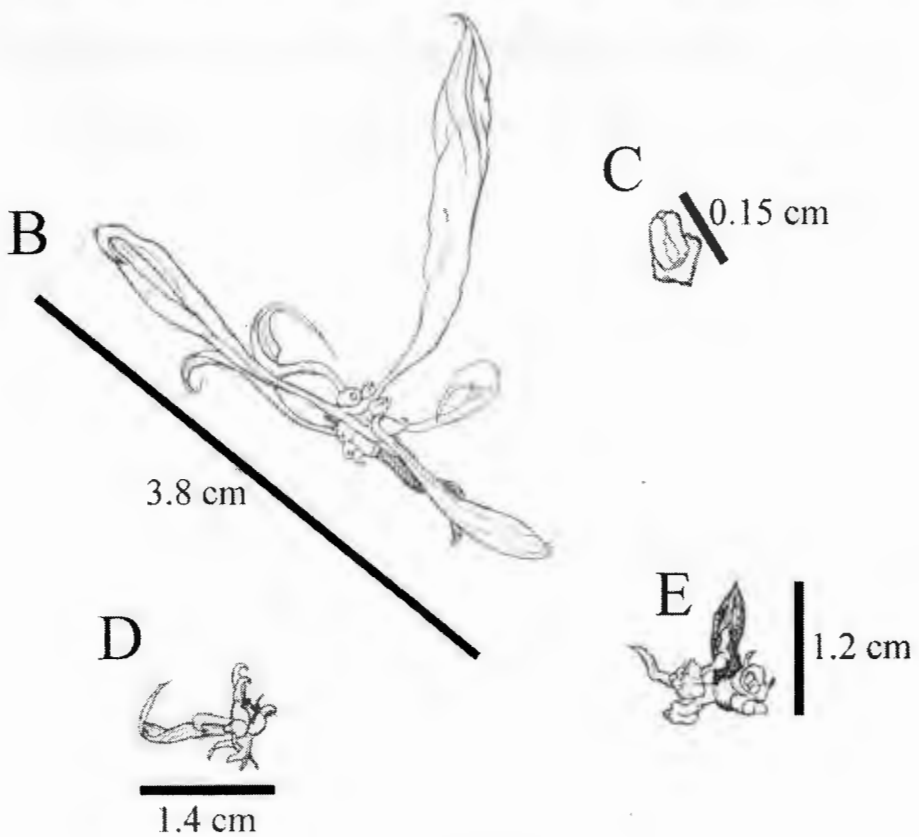
C - Small protocorm-like body separated from plantlet in Fig. 1A.

D - Protocorm-like body showing a cluster of small pitchers.

E - Protocorm-like body with large emerging leaf.

All drawings by Leslie Anne Uhnak.


A


B
3.8 cm
C
0.15 cm
D
1.4 cm
E
1.2 cm

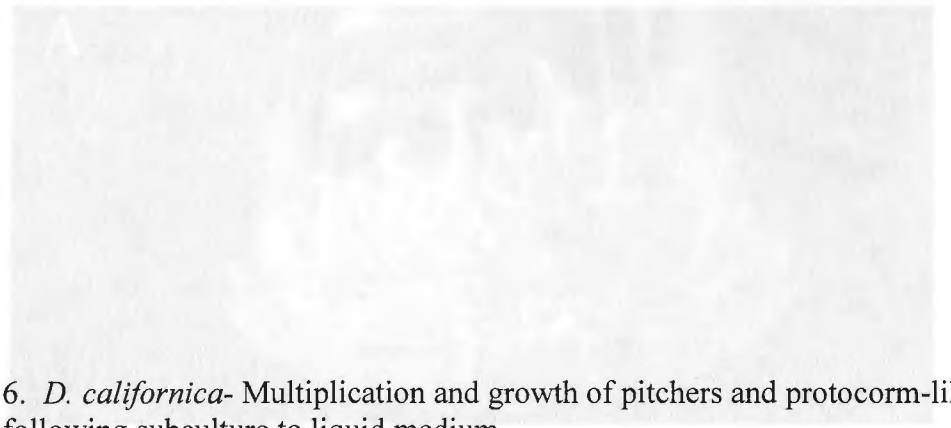


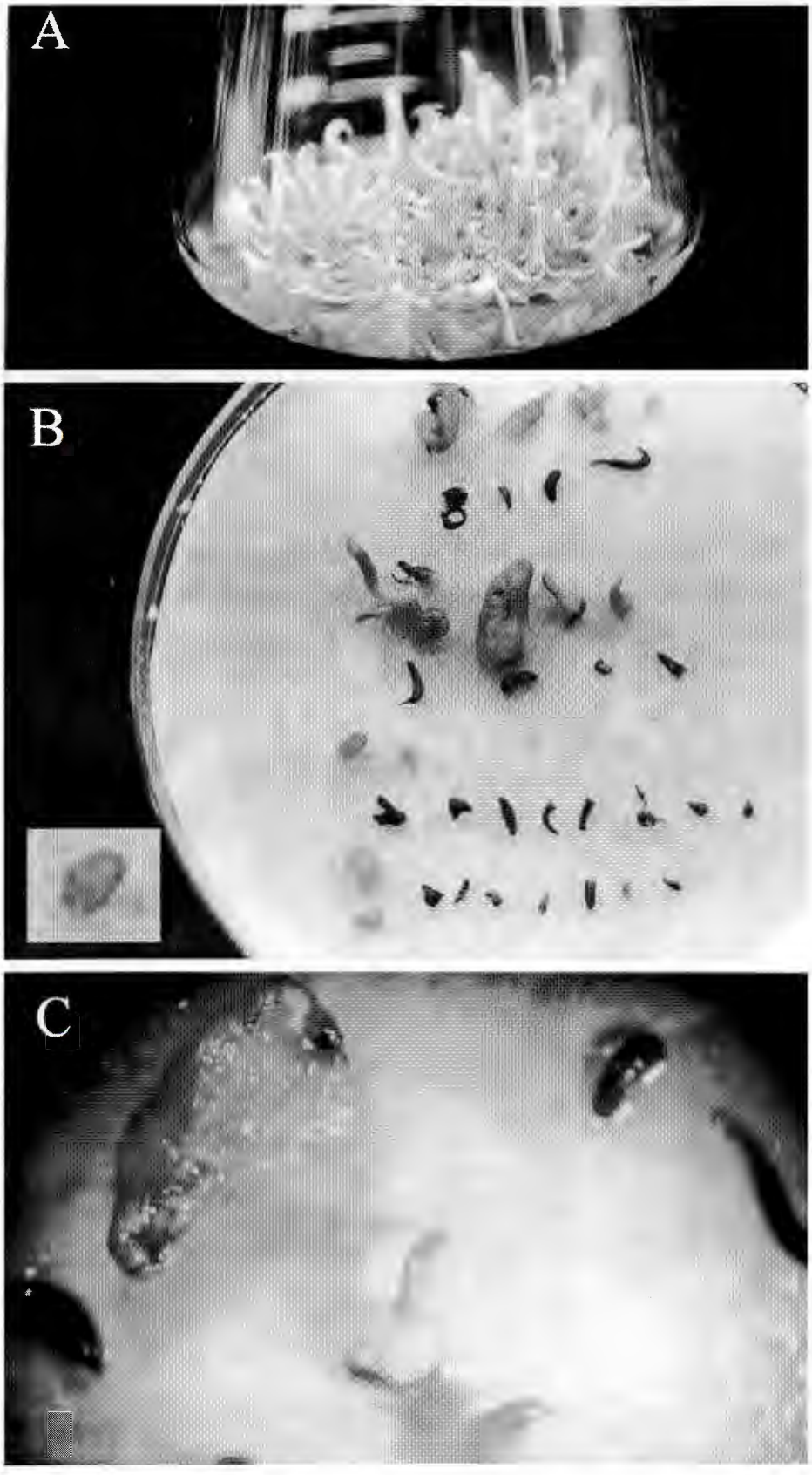
Figure 6. *D. californica*- Multiplication and growth of pitchers and protocorm-like bodies following subculture to liquid medium.

A – Multiplication of 6 clusters of pitchers after 45 days of subculture in liquid POMM (X1.0)

B - Protocorm-like bodies and callus produced from fragmented pitchers grown in liquid POMM (X1.3)

B inset - Small spherical body produced from pitcher fragment (X10)

C - Close-up of PLB in Fig. 6A(X 3.5) (compare to Fig. 1D).


A
B
C

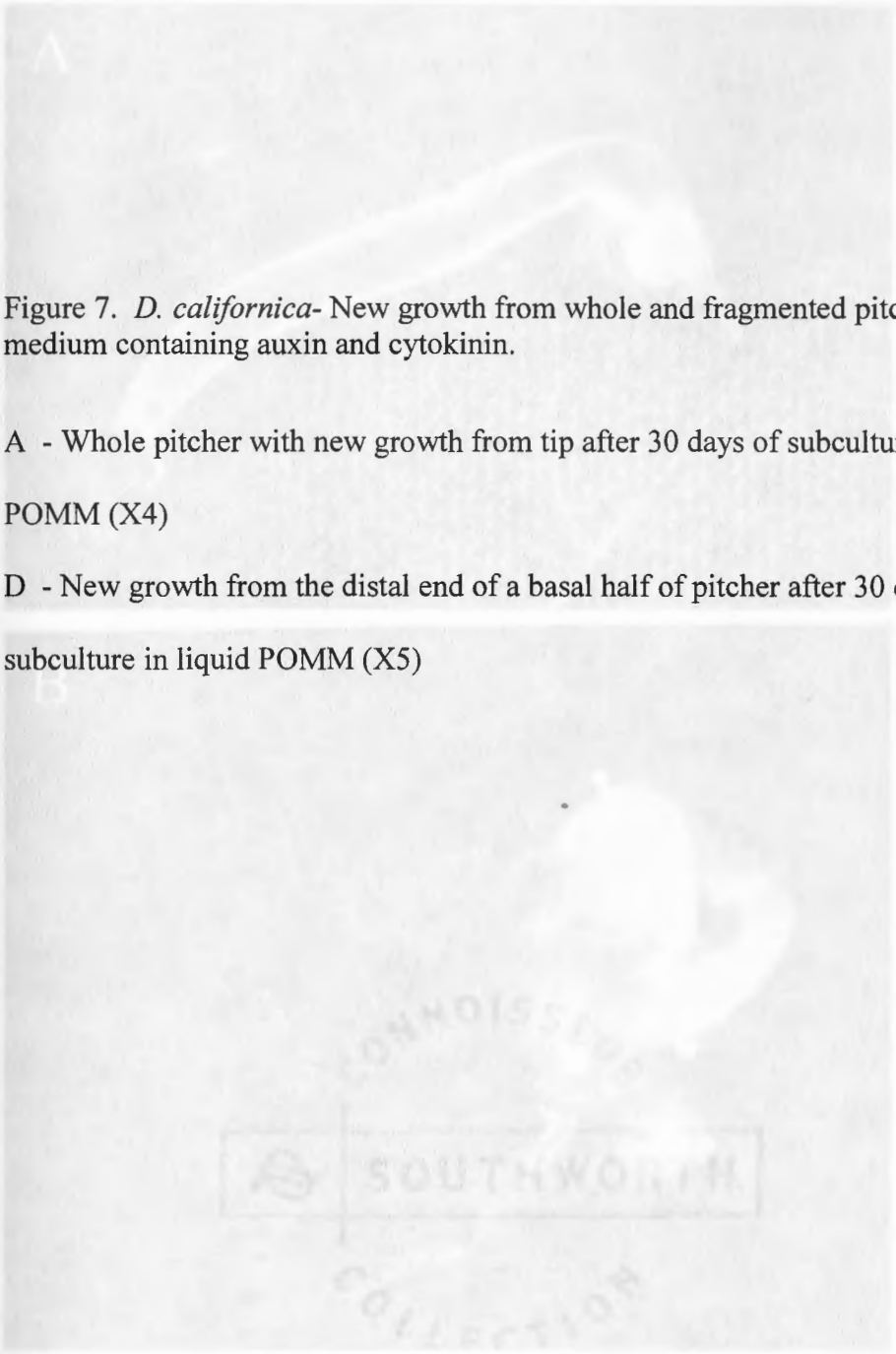


Figure 7. *D. californica*- New growth from whole and fragmented pitchers in liquid medium containing auxin and cytokinin.

A - Whole pitcher with new growth from tip after 30 days of subculture in liquid POMM (X4)

D - New growth from the distal end of a basal half of pitcher after 30 days of subculture in liquid POMM (X5)

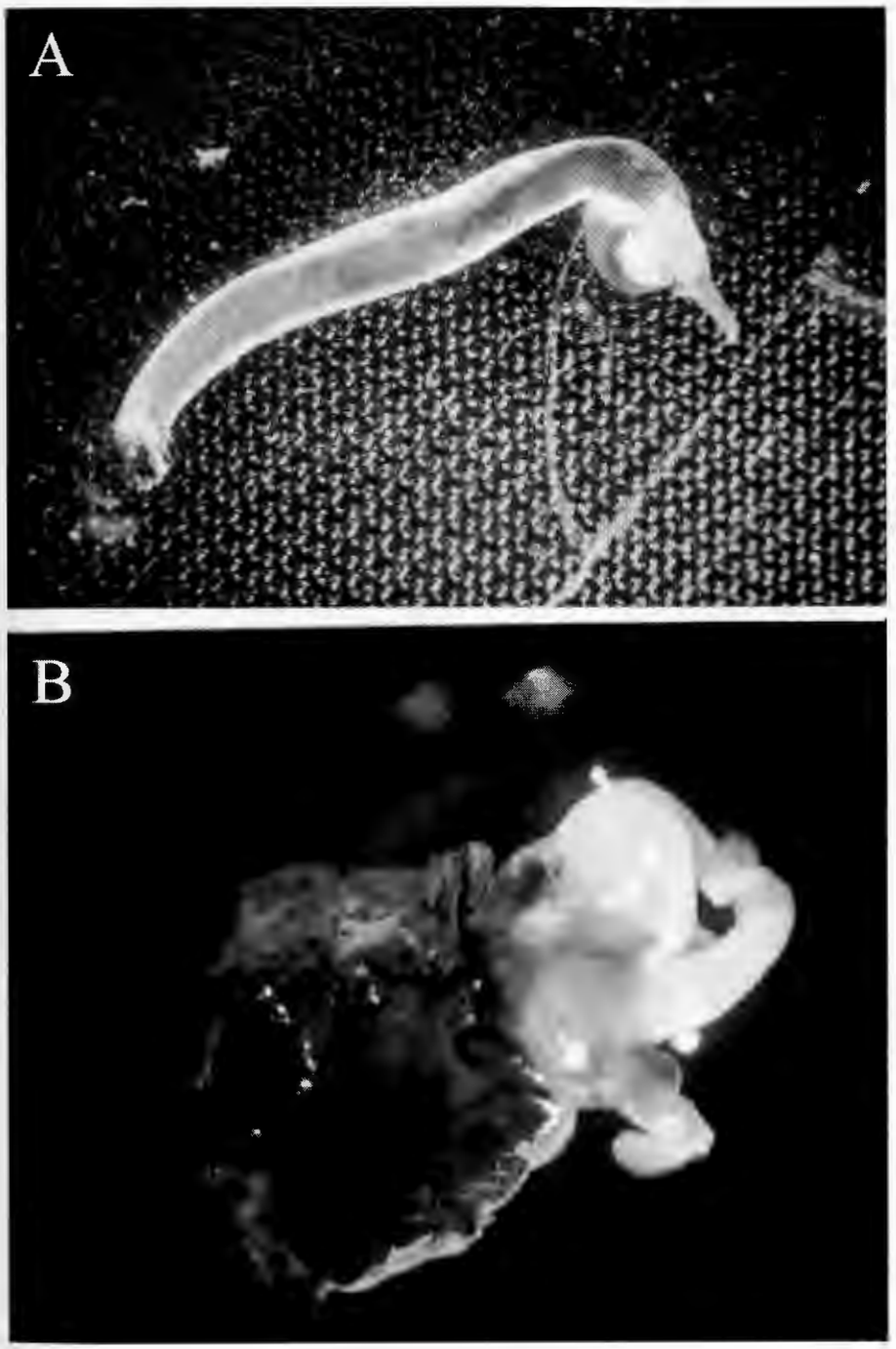
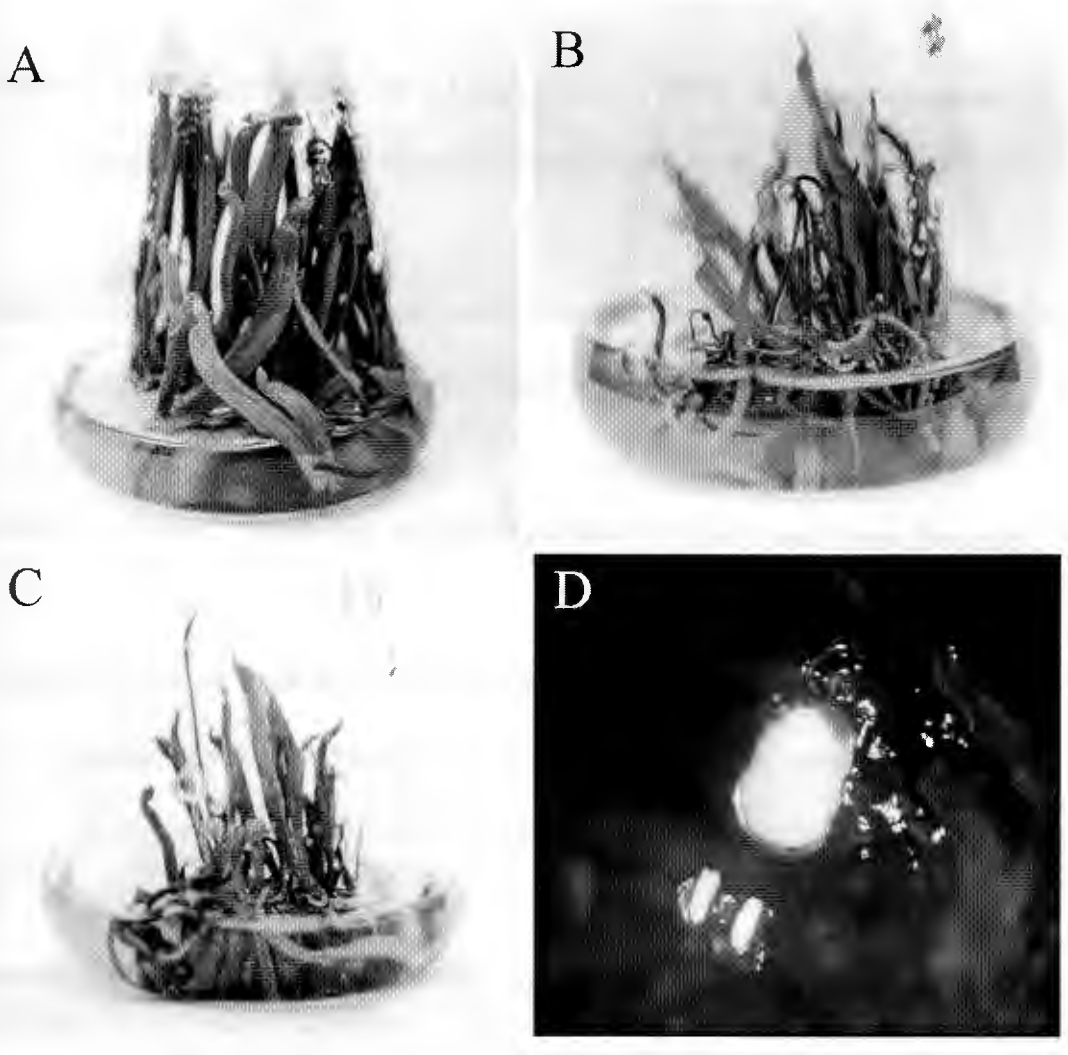

A
B

Figure 8. *S. leucophylla* –Seedlings, endosperm and embryos.

A-C - Seedlings of *S. leucophylla* after 6 months of subculture *in vitro* (Fig. 8A X0.7; Fig. 8B X0.9; Fig. 8C X0.8)

D - Two extracted embryos are shown along with a seed of *S. leucophylla* from which the seed coat has been removed to reveal yellow-white endosperm (X12)


A
B
C
D

Literature Cited

Adamec L (1997) Mineral Nutrition of Carnivorous Plants: A Review. Bot. Rev. 63: 273-299

Adams II, R.M., Koenigsberg SS & Langhans RW (1979) *In vitro* propagation of *Cephalotus follicularis* (Australian pitcher plant). HortScience 14(4): 512- 513

Cheers G (1992) A Guide to Carnivorous Plants of the World. New York. (pp. 60-61) Angus & Robertson, Harper Collins Publishers. Australia

D'Amato P (1998) The Savage Garden. (pp.100-103) Ten Speed Press. Berkeley, CA

Franck DH (1975) Early histogenesis of the adult leaves of *Darlingtonia californica* (Sarraceniaceae) and its bearing on the nature of epiascidiate foliar appendages. Am. J. Bot. 62: 116-132

Franck DH (1976) Comparative morphology and early leaf histogenesis of adult and juvenile leaves of *Darlingtonia californica* and their bearing on the concept of heterophylly. Botanical Gazette, 137: 20-34

Givnish TJ (1989) Ecology and evolution of carnivorous plants. Plant-Animal Interactions. (pp. 243-290) McGraw-Hill Inc. New York.

Godt MJW & Hamrick JL (1996) Genetic structure of two endangered pitcher plants, *Sarracenia jonseii* and *S. oreophila* (Sarraceniaceae). Am. J. Bot. 83(8): 1016-1023

Hu CY & Wang PJ (1983) Meristem, Shoot Tip and Bud Cultures. In: Handbook of Plant Cell Culture. (pp.177-227) Evans, D.A., Sharp, W.R., Ammirato, P.V & Yasuyuki, Y. (eds) Macmillan Publishing Co., New York.

Kohlenbeck HW (1978) Comparative Somatic Morphogenesis. In Frontiers of Plant Tissue Culture. Thorpe TA (ed) Published by International Association for Plant Tissue Culture. Alberta, Canada

Lane WD (1979) The influence of growth regulators on root and shoot initiation from flax meristem tips and hypocotyles *in vitro*. Physiol. Plant. 45: 260-264

Lloyd EF (1942) The Carnivorous Plants. (pp.17-39) Chronica Botanica Co. Waltham, MA.

MacDougal DT (1903) The influence of light and darkness upon growth and development. Mem. New York Bot. Gard. 2: 1-319

Malik KA & Saxena PK (1991) Regeneration in *Phaseolus vulgaris* L.: High frequency induction of direct shoot formation in intact seedlings by N^6-benzylaminopurine and thidiazuron. Planta 186: 384-389

Morel G (1974) Clonal Multiplication of Orchids. In: The Orchids, Scientific Studies. (pp. 169-222) Withner CL (ed) Wiley-Interscience, New York

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497

Parliman BJ, Evans PT & Rupert EA (1982a) Tissue culture of single rhizome explants of *Dionaea muscipula* Ellis ex. l., the venus fly trap, for rapid asexual propagation. J. Am. Soc. Hort. Sci. 107 (2): 305-310

Parliman BJ, Evans PT & Mazur AR (1982b) Adventitious bud differentiation and development in leaf cuttings of D*ionaea muscipula* Ellis ex. l. (venus fly trap) cultured *in vitro*. J. Am. Soc. Hort. Sci. 107 (2): 310-316

Pietropaolo J & Pietropaolo P. (1986) Carnivorous Plants of the World. (pp. 38-39) Timber Press. Portland, Oregon

Rathore TS, Tandon P & Shekhawat NS (1991) *In vitro* regeneration of pitcher plant (*Nepenthes khasiana* Hook. f.) – a rare insectivorous plant of India. J. Plant Physiol. 139: 246-248

Redwood G & Bowling D (1990) Micropropagation of *Nepenthes* species. Bot. Gard. Microprop. News 1 (2): 19-20

Schnell DE (2002). Carnivorous Plants of the United States and Canada. (pp. 230-233) Second Edition. Timber Press. Portland, Oregon

Teo WL, Kumar P, Goh C & Swarup S 2001. The expression of *Brostm*, a *KNOTTED1*-like gene, marks the cell type and timing of *in vitro* shoot induction in *Brassica oleracea*. Plant Mol. Biol. 46: 567-580

Truscott FH (1966) Some aspect of morphogenesis *in Cuscuta gronovii*. Am. J. Bot. 53 (7): 739-750

Walker DB (1975) Postgenital carpel fusion in *Catharanthus roseus* (Apocynaceae). I. Light and scanning electron microscopic study of gynoecial ontogeny. Am. J. Bot. 62(5): 457-467

APPENDIX A

RESULTS ANCILLARY TO MANUSCRIPT I

Surface Disinfestation Study – Controls

Darlingtonia californica - Sterile H_2O + Surfactant 3 x 5 min washes in sterile distilled water + 1 drop of Tween 20 DCW1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)	
	Sterile Wash
Contamination after 15 days	17 of 48 seeds contaminated by day 15 (fungal mycelia present in all 17)

Darlingtonia californica - Sterile H_2O + Surfactant 3 x 5 min washes in sterile distilled water + 1 drop of Tween 20 DCW2 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)	
	Sterile Wash
Contamination after 15 days	1 of 48 seeds contaminated by day 15 (fungal mycelium)

Sarracenia leucophylla- Sterile H_2O + Surfactant 3 x 5 min washes in sterile distilled water + 1 drop of Tween 20 SLW1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)	
	Sterile Wash
Contamination after 15 days	48 of 48 seeds contaminated by day 7 (41 fungal mycelia, 7, yeast/bacterial)

Sarracenia purpurea - Sterile H_2O + Surfactant 3 x 5 min washes in sterile distilled water + 1 drop of Tween 20 SPW1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)	
	Sterile Wash
Contamination after 15 days	45 of 48 seeds contaminated by day 15 (early bacterial contamination of many seeds)

Sarracenia alata - Sterile H_2O + Surfactant 3 x 5 min washes in sterile distilled water + 1 drop of Tween 20 SPW1 24 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)	
	Sterile Wash
Contamination after 15 days	24 of 24 seeds contaminated by day 7 (fungal mycelia)

Sarracenia rubra - Sterile H_2O + Surfactant 3 x 5 min washes in sterile distilled water + 1 drop of Tween 20 SRW1 24 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)	
	Sterile Wash
Contamination after 15 days	24 of 24 seeds contaminated by day 15 (fungal mycelia)

Results of Surface Sterilization Experiments

Darlingtonia californica 3% Hydrogen Peroxide DCHP1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	0 of 48	0 of 48	0 of 48	1 of 48 (2.1%)

Darlingtonia californica 3% Hydrogen Peroxide DCHP2 48 seeds – one seed per well in plastic well plates (MYP + 10g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	0 of 48	0 of 48	0 of 48	0 of 48

Darlingtonia Californica Experiment #DCCH1 and DCCH2 - 10% Clorox + surfactant

Darlingtonia californica 10% Clorox DCCH1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	0 of 48	0 of 48	0 of 48	0 of 48

Darlingtonia californica 10% Clorox DCCH2 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	0 of 48	0 of 48	0 of 48	0 of 48

Darlingtonia californica – Concentrated H_2SO_4 DCCS1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	0 of 48	0 of 48	0 of 48	0 of 48

Darlingtonia californica - Concentrated H_2SO_4 DCCS2 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	0 of 48	0 of 48	0 of 48	0 of 48

Darlingtonia californica 1.5% Physan 20 DCPhy1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	0 of 48	0 of 48	0 of 48	0 of 48

Darlingtonia californica 1.5% Physan 20 DCPhy2 48 seeds – one seed per well in plastic well plates (MYP + 10g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	0 of 48	0 of 48	0 of 48	0 of 48

Sarracenia leucophylla 3% Hydrogen Peroxide SLHP1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	31 of 48 (65.0%)	48 of 48 by day 6	24 of 48 (50.0%)	21 of 48 (44.0%)

Sarracenia leucophylla 10% Clorox SLCH1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	8 of 48 (17.0%)	4 of 48 (8.3%)	45 of 48 (93.0%)	4 of 48 (8.3%)

Sarracenia leucophylla – Concentrated H_2SO_4 SLCS1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	25 of 48 (52.1%)	19 of 48 (40.0%)	7 of 48 (14.6%)	3 of 48 (6.3%)

Sarracenia leucophylla - Concentrated H_2SO_4 SLCS2 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	27 of 48 (56.3%)	22 of 48 (45.8%)	12 of 48 (25%)	5 of 48 (10.4%)

Sarracenia leucophylla 1.5% Physan 20 SLPhy1 48 seeds – one seed per well in plastic well plates (MYP + 10 g/l sucrose (pH 6.5)				
	4 min	8 min	12 min	16 min
Contamination after 15 days	44 of 48 (91.7%)	33 of 48 (68.8%)	32 of 48 (66.6%)	30 of 48 (62.5%)

Liquid Germination Experiments – *Sarracenia leucophylla*

Sarracenia leucophylla Experiment SL21 Type of Experiment - Germination Disinfestation – 10 minutes in H_2SO_4 -Treatments - 6 seeds/flask, 6 flasks/treatment Temp and light – Room conditions

	Water (ph. 5.0)							½ Strength MS					
Flask #	1	2	3	4	5	6		1	2	3	4	5	6
Day													
10	1	1	cont	-	-	1		-	-	-	-	-	-
11	1			-	-			-	-	-	-	-	
12	1	2		-	-	2		-	-	-	-	-	-
13	1	2		-	-	2		-	-	-	-	-	1
14	1	2		-	-	2		-	-	-	-	-	1
15	1	cont		-	-	2		-	-	-	-	-	1
16	1	2		-	-	2		-		-	-	-	1
17	1	2		-	-	2		-	-	-	-	-	1
18				-	-			-	-	-	-	-	
19	1	2		-	-	2		-	-	-	-	-	1
20	1	2		-	-	2		-	-	-	-	-	1
21	1	2		-	-	2		-	-	-	-	-	1
22	1	2		-	-	2		-	-	-	-	-	1
23	1	2		-	-	2		-	-	-	-	-	1

Total germinated = 3 of 24 = 12.5% Total Germinated = 1 of 36 = 2.7%

Results of Experiment # SL1R1 *Sarracenia leucophylla* - Type of Experiment- Germination - seeds not stratified - 20 minutes in 10% Clorox - 6 seeds/flask – 6 flasks/treatment 50 ml Erlenmeyer on gyrotary shaker – room light and temperature conditions – Start date 2/28/02.

Medium - Water (pH 5.0)

Flask #	1	2	3	4	5	6
Day	*	*	*		*	*
15		2		2		
16	1	2		2		2
17	1	2	1	3	1	2
18						
19						
20						
21	1	2	1	3	1	2

Total germinated = 3 of 6 = 50%

Results of Experiment # SL2R1 *Sarracenia leucophylla* - Germination - seeds not stratified - 10 minutes in concentrated H_2SO_4 - 6 seeds/flask – 6 flasks/treatment 50 ml Erlenmeyer on gyrotary shaker – room light and temperature conditions – Start date 2/02/02 (Note: Flasks 1, 4, and 7 contained 7 seeds.)

Flask #	1	2	3	4	5	6
Day	*	*	*	*	*	
11	4	2	5	3	3	3
12						
13	5	2	5	3	3	3
14	5	2	5	3	3	6
15						
16						
17						
18						
19						
20						
21	5	2	5	3	3	6 of 7

Medium - Water (pH. 5.0)

Total germinated = 6 of 7 = 85.7%
* = contamination

Sarracenia leucophylla - Seed Selection Experiments – When seeds obtained from a commercial source were inspected with the aid of a dissecting microscope they could be divided into four categories; 1) Normal or non-suspect seeds – these seeds exhibit normal morphology, light brown color, outer coat heavily waxed, and no surface detectable imperfections; 2) Infected seeds – these seeds exhibit large areas of seed coat covered by a colored mass, usually gray, green, yellow or orange red, that appears to be tightly adhering fungal hyphae or sporulating structures. They may also have small holes in the seed coat; 3) Suspect seeds – these seeds appear to be normal as far as their general appearance, however on closer inspection, some cells of the outer seed coat may appear black or discolored. These areas may comprise several cells in diameter and can go undetected if the seed is not carefully examined; 4) Abnormal seeds – these seeds present abnormal developmental morphology and they may be very small, round, cup shaped or they can be elongate and narrow. Seeds were divided into the four categories and 20 seeds from each category were weighed.

	Normal	Infected	Suspect	Abnormal
Weight of 20 seeds	0.0170g	0.0134g	0.0149g	0.0010g
Average weight of 1 seed	0.00085g	0.00067g	0.000745	0.00005g

Results of Experiment # SL1R2 – Germination – *Sarracenia leucophylla* - seeds not stratified - 10 minutes in concentrated H_2SO_4 with frequent mixing on vortex mixer and gentle agitation with tip of Pasteur pipet – H_2O_2 rinse on vortex mixer- rinsed in sterile H_2O- solid and liquid treatments - liquid treatments in 50 ml Erlenmeyer flasks – 6 flasks- 6 seeds per flask -on gyrotary shaker at room temp and light. Solid treatment in 250 ml Erlenmeyer flasks – 3 flasks – 12 seeds/flask – room temperature and light. Start date 4/10/02.

Treatment #1 – 75 ml of solid ½ strength MS medium + vitamins

Flask # 1	Flask # 2	Flask # 3
1 seed contaminated on day 11	1 seed contaminated on day 5	1 seed contaminated on day 11
No germination after 21 days	No germination after 21 days	No germination after 21 days

Results of Experiment # DC1R1 *Darlingtonia californica* - Type of Experiment- Germination - seeds not stratified - 10 minutes in 10% Clorox - 6 seeds/flask – 6 flasks/treatment 50 ml Erlenmeyer - on gyrotary shaker – room light and temperature conditions – Start Date 2/28/02

Medium - Water (pH 5.0)

Flask #	1	2	3	4	5	6
Day						
16	4	4	4	4	4	6
17	5	6	6	5	6	6
18	5	6	6	5	6	6
19						
20						
21	5	6	6	5	6	6

Total germinated = 34 of 36 = 94.4%

Results of Experiment # DCCUTT #1 *Darlingtonia californica* - Germination - seeds not stratified - 10 minutes in H_2O_2 - 30 seeds were cut across the micropylar end and the distal portion removed - seeds were plated on water agar (pH 5.0) in a petri dish – room light and temperature conditions – Start Date 5/2/02

Day	# of Seeds Germinated	Percent Germination
13	15	50%
14		
15	18	60%
16		
17	20	66.6%
18		
19	21	70%
20		
21	23	76.7%

Germination = 76.7% in 21 days - Germination began on day 13

Results of Experiment # DCCUTT #2 *Darlingtonia californica* - Type of Experiment Germination - seeds not stratified - 10 minutes in H_2O_2 - 30 seeds were cut across the micropylar end and the distal portion removed - seeds were plated on water agar (pH 5.0) in a petri dish – room light and temperature conditions – Start Date 5/2/02

Day	# of Seeds Germinated	Percent Germination
13	15	50%
14		
15	18	60%
16		
17	20	66.6%
18		
19	21	70%
20		
21	23	76.7%

Germination = 76.7% in 21 days - Germination began on day 13

Results of Experiment DCGEM2002 – Seeds used in this experiment were given three different treatments before plating on three different substrates. One hundred and twenty seeds of *D. californica* were removed from storage at 4-7°C and surface sterilized. Seeds were soaked overnight in 10 ml of sterile distilled H_2O (pH.5.0) + 1 drop of Tween 20 at 4-7°C.

Another 120 seeds were soaked under the same conditions except the H_2O solution contained 6 mg/l GA_3. Surface disinfestation was in 3% H_2O_2 for 10 minutes for all seeds. Seeds were not rinsed before being plated onto petri dishes containing - water agar (pH 5.0) – wet filter paper (pH 5.0) – filter paper wet with H_2O containing 6 mg/l GA_3. - 36 seeds/petri dish. Start date – May 1, 2002.

Treatment 1 – Seeds not soaked

Day	Treatment Water agar (pH 5.0)	Treatment Wet filter paper (pH 5.0)	Wet filter paper + 6 mg/l GA_3
10			
11			
12			
13	4	0	0
14			
15	18	0	0
16			
17	25	0	2
18			
19	30	0	2
20			
21	31	0	2
	86.1% germination	0% germination	5.5% germination

Treatment 2 – Seeds soaked overnight in H_2O + surfactant

Day	Treatment Water agar (pH 5.0)	Treatment Wet filter paper (pH 5.0)	Wet filter paper + 6 mg/l GA_3
10			
11			
12			
13	21	0	0
14			
15	26	0	0
16			
17	28	1	0
18			
19	29	1	2
20			
21	30	1	2
	83.3% germination	2.8 % germination	5.5% germination

Treatment 3 – Seeds soaked overnight in H_2O +surfactant + 6 mg/l GA_3

Day	Treatment Water agar (pH 5.0)	Treatment Wet filter paper (pH 5.0)	Wet filter paper + 6 mg/l GA_3
10	7		1
11			
12			
13	18	0	2
14			
15	20	0	7
16			
17	27	1	9
18			
19	31	2	10
20			
21	32	2	10
	88.9% germination	2.8 % germination	27.8% germination

Darlingtonia californica - Seed weights from a batch obtained in February 2002, which showed good germination. Batch was weighed immediately after removal from storage at 4-7°C.

February Batch

	Weight of 12 seeds	Weight of 24 seeds
1	0.0022g	0.0039g
2	0.0021g	0.0043g
3	0.0022g	0.0044g
4	0.0018g	0.0041g
5	0.0020g	0.0040g
6	0.0020g	0.0046g
7	0.0022g	0.0042g
8	0.0023g	0.0043g
9	0.0022g	0.0042g
10	0.0020g	0.0042g
	Average weight per seed = 0.000175g	Average weight of each seed =0.000178g

Results of Experiment DC51 and DC52 – *Darlingtonia californica*

Day	Flask # 1	2	3	4	5	6	1	2	3	4	5	6
10	2	3	2	1	3	3			*	*	1	1
11												
12	4	4	2	3	3	3					1	1
13	5	4	3	3	4	4					1	1
14	5	4	4	3	4	4					1	1
15	6	4	4	3	4	4					1	1
16	6	4	4	3	6	4					1	1
17	6	4	4	3	6	4	1				1	1
18												
19	6	4	4	4	6	4	2				1	2
20	6	4	4	4	6	4	2				1	2
21	6	4	4	4	6	4	2	1	0	0	2	2
28 of 36 seeds germinated in 21 days Germination = 77.8%							6 of 36 seeds germinated in 21 days Germination = 16.7 %					

10 minutes 10% Clorox – 6 seeds/flask – 6 x 50 ml flasks – gyrotary shaker- room light and temp. Start date 4/10/02

Treatment # 1 H_2O DC52 Treatment # 2 liquid ½ strength MS

Day	Flask # 1	2	3	4	5	6	1	2	3	4	5	6
10	2	1	3	1	2	2			1			1
11												
12	3	5	3	1	3	2			2			1
13	4	5	4	2	3	2			2			1
14	4	5	4	2	3	2			2			1
15	5	5	4	2	3	2			2		1	1
16	5	5	4	2	3	2	2	*	2	1	3	1
17	5	5	4	2	4	3	2		2	1	4	1
18												
19	6	5	4	2	5	3	2		3	2	4	1
20	6	5	5	2	5	3	2		3	2	4	1
21	6	5	5	2	5	3	2		3	2	4	1
26 of 36 seeds germinated in 21 days Germination = 66.7%							12 of 36 seeds germinated in 21 days Germination = 33.3 %					

* = contamination

Germination in the Dark

Results of The Dark Experiments – These experiments were conducted whenever more seeds than were needed for an experiment were surface sterilized. Excess seeds were plated on available media and placed in the dark at 27°C and observations made over various time periods.

Dark Experiment A1 - May 2, 2002 – Eighteen seeds of *D. californica* were treated overnight in H_2O (pH.5.0) + 1 drop of Tween 20 , surface sterilized for 10 min in H_2O_2, and plated on H_2O agar (pH.5.0). No germination as of May 17, 2002 (15 days) however, seeds from experiment # DCGEM2002 started on may 1 (room temp and light) had 50% germination on May 15. No germination as of May 30, 2002. Seeds were next observed on June 15, 2002 and 3 seeds had germinated. This experiment was terminated on July 15, 2002. A total of 9 seeds had germinated in 3 months.

Dark Experiment A3 – Excess seeds (22) from DC51 treatment #1 were plated on water agar and stored in the dark at 27°C. No germination as of day 22 – 2 germinated on day 23.

Dark Experiment A3 - For purposes of an initial comparison with the above experiments, extra seeds (*D. californica*) from experiment # DCGEM2002C were plated on water agar and placed in the growth chamber at 27°C with a 16 hour photoperiod.

Results:

Day	Treatment #1 – not stratified (44 seeds)	Treatment #2 – 24 hour soak in H_2O (9 seeds)	Treatment #3 – 24 hour soak in H_2O +GA_3 (37 seeds)
13	7 of 44 germinated	4 of 9 germinated	29 of 37 germinated
21	8 of 44 germinated	5 of 9 germinated	33 of 37 germinated

Germination Results from a Batch Received in Feb. 2002 – *Darlingtonia californica*

Results of Experiment DCGEM2002C – Germination (repeat of DCGEM2002) – Water Agar (pH.5.0) – 3 pretreatments – Not stratified - 24 hr soak in H_2O – 24 soak in GA_3 – surface sterilization in H_2O_2 10 min. – Batch from Feb 2002 – 25 seeds per petri dish - Start Date – 7/23/02

	Germination – not stratified - water agar - pH 5.0 (25 seeds/plate)			Germination following 24hr soak in H_2O water agar - pH 5.0		
Day	Plate #1	Plate #2	Plate#3	Plate #1	Plate #2	Plate #3
12	2	0	0	4	5	6
13	4	1	1	5	7	8
14	5	2	3	5	8	10
15	5	3	4	6	9	10
16						
17	6	4	4	6	10	10
18						
19						
20	6	5	5	10	11	12
21	6	5	5	11	11	13
	Germination - 16 of 75 seeds after 21 days			Germination - 35 of 75 seeds after 21 days		

	Germination – 24 hour soak in GA_3 water agar - pH 5.0 (25 seeds/plate)		
Day	Plate #1	Plate #2	Plate #3
12	10	8	7
13	12	12	8
14	13	12	9
15	14	13	9
16			
17	16	15	11
18			
19			
20	18	19	13
21	20	19	17
	Germination - 56 of 75 seeds after 21 days		

Germination Results from a Batch Received in Feb. 2002 – *Darlingtonia californica*

Results of Experiment DCGEM2002C – Germination (repeat of DCGEM2002) – Water Agar (pH.5.0) – 3 pretreatments – Not stratified - 24 hr soak in H_2O – 24 soak in GA_3 – surface sterilization in H_2O_2 10 min. – Batch from Feb 2002 – 25 seeds per petri dish - Start Date – 7/23/02

	Germination – not stratified - water agar - pH 5.0 (25 seeds/plate)			Germination following 24hr soak in H_2O water agar - pH 5.0		
Day	Plate #1	Plate #2	Plate#3	Plate #1	Plate #2	Plate #3
12	2	0	0	4	5	6
13	4	1	1	5	7	8
14	5	2	3	5	8	10
15	5	3	4	6	9	10
16						
17	6	4	4	6	10	10
18						
19						
20	6	5	5	10	11	12
21	6	5	5	11	11	13
	Germination - 16 of 75 seeds after 21 days			Germination - 35 of 75 seeds after 21 days		

	Germination – 24 hour soak in GA_3 water agar - pH 5.0 (25 seeds/plate)		
Day	Plate #1	Plate #2	Plate#3
12	10	8	7
13	12	12	8
14	13	12	9
15	14	13	9
16			
17	16	15	11
18			
19			
20	18	19	13
21	20	19	17
	Germination - 56 of 75 seeds after 21 days		

Germination Results from a Batch Received in June 2002 – *Darlingtonia californica*

Results of Experiment DCGEM2002B – Germination (seedlings for research) – Water Agar and sphagnum agar - 24 hr soak H_2O and also GA_3 - H_2O_2 10 min. – Batch from June 2002 – 40 seeds per petri dish - Start Date – 7/23/02 Data taken Sept. 8, 2002.

	Germination following 24hr soak in H_2O water agar - pH 5.0			Germination following 24hr soak in H_2O + 6 mg/l GA_3 water agar - pH 5.0		
Day	Plate #1	Plate #2	Plate#3	Plate #1	Plate #2	Plate #3
47	1	3	5	4	1	4
	9 of 120 (40 seeds/plate)seeds = 7.5% germination			9 of 120 (40 seeds/plate) seeds = 7.5% germination.		

	Germination following 24hr soak in H_2O Sphagnum based agar - pH 5.0			Germination following 24hr soak in H_2O + 6 mg/l GA_3 Sphagnum based agar - pH 5.0		
Day	Plate #1	Plate #2	Plate#3	Plate #1	Plate #2	Plate #3
47	0	0	0	0	0	0
	0% germination after 47 days (120 seeds)			0% germination after 47 days (120 seeds)		

Results of Exp. GESD - Germination (seedlings for research) – Water agar - 24 hr soak H_2O - H_2O_2 10 min. - room temp and light – Batch from June 2002 – 25 seeds per petri dish - Start Date – 8/1/02 Data taken Sept. 8, 2002.

Germination following 24hr soak in H_2O - water agar pH 5.0								
Day	Plate #1	Plate #2	Plate #3	Plate #4	Plate #5	Plate #6	Plate #7	Plate #8
38	1	1	0	1	1	1	2	0
7 of 200 seeds germinated in 38 days = 3.5% germination								

Plates from GESD were placed in the growth chamber on 9/8/02

RESULTS ANCILLARY TO MANUSCRIPT II

Results of Experiment M1R1 - Growth on solid ½ Strength MS Medium + Vitamins

Tube #	No. of Pitchers	Length of Pitchers (mm)	Roots	Color	Observations
1	6	11,9,7,10 ,8,8	Primary	Green	
2	6	10,10,11, 8,9,7	Primary	Green	Primary root dichotomously split at tip
3	5	8,8,6,5,7	Primary, 1 secondary	Green	
4	7	12,11,11, 3,4,4, 9	Primary	Green	
5	5	6,8,7,4,3	Primary	Green	
6	6	6,3,3,4,8, 7	Primary	Green	
7	4	5,4,4,3	Primary	Green	
8	6	10,10,9,4 ,7,8	Primary, 1 early secondary	Green	
9	7	12,12,11, 7,4,9,8	Primary	Green	Primary very well developed
10	5	6,5,2,3,8	Primary		
11	6	6,6,7,5,4, 3	Primary	Green	
12	4	8,7,5,3	Primary	Green	
13	3	5,4,3	Primary	Green	
14	6	3,3,4,7,6, 6	Primary	Green	
15	6	12.12.11. 9.10,4	Primary, 1 well developed secondary	Green	Secondary arising from rhizome
16	5	6,5,5,4,3	1 Primary	Green	
17	5	5,4,5,3,3	1 Primary	Green	
18	6	12,11,11, 9,7,5	1 Primary	Green	

Total dry Mass = 0.0370

Results of Experiment M1R1 – Growth on Solid Sphagnum-based Medium + Vitamins

Tube #	No. of Pitchers	Length of Pitchers (mm)	Roots	Color	Observations
1	3	4,3,3	Primary	Light green	
2	5	5,4,4,3,3	Primary	Light green	
3	3	4,3,3	Primary	Yellow	
4	5	5,6,4,3,3	Primary	Yellow Green	
5	4	5,5,4,3	Primary	Yellow	
6	5	5,3,3,3,4	Primary	Yellow green	
7	4	3,5,5,3	Primary	Yellow green	
8	56	5,5,4,4,6,3	Primary with secondary	Green	
9	2	4,3	Primary	Yellow brown	Stopped development appr. week 2
10	5	6,5,4,4,4	Primary	Green	
11	2	3,3	Primary	Yellow brown	Stopped development appr. week 2
12	2	3,4	Primary	Yellow brown	
13	3	3,3,4	Primary	Yellow green	
14	6	6,6,5,4,4,3	Primary	Green	
15	4	4,3,3,3	Primary	Yellow green	
16	5	6,5,5,4,3	Primary with secondary	Green	
17	6	6,6,3,5,5	Primary with Bud	Yellow green	
18	5	5,4,4,3,3	Primary	Yellow green	

Total dry Mass = 0.0195

Results of Experiment M2R2 – Growth on Solid ½ Strength MS + Vitamins

Results of Experiment M2R2 – Growth on Solid ½ Strength MS + Vitamins

Tube #	No. of Pitchers	Length of Pitchers (mm)	Roots	Color	Observations
1	7	5,6,4,4,6,3,3	Primary with 1 bud	Green	
2	3	3,4,3	Primary	Brown	Stopped development appr. week 2
3	8	5,6,6,5,4,4,5,4	Primary with 1 bud	Green	
4	9	9,8,8,7,8,6,5,5,4	Primary with 1 bud	Green	
5	9	10,10,9,9,8,7,9, 6,5	Primary with 2 buds	Green	
6	5	5,6,5,4,3	Primary	Green	
7	6	7,8,7,6,4,4	Primary with 1 bud	Green	
8	3	4,4,3	Primary	Green	
9	7	6,7,6,5,4,4,4	Primary split dichotomously at tip	Green	
10	5	7,7,6,5,5	Primary	Green	
11	8	12,13,12,10.9.8. 7.8	Primary with 2 buds	Green	
12	8	9,8,8,7,6,5,7,8	Primary with 2 bud	Green	
13	5	6,5,5,4,4	Primary	Green	
14	3	4,5,3	Primary	Yellow brown	Stopped development appr. week 2
15	5	5,4,6,6,5	Primary with 2 buds	Green	
16	5	5,4,4,4,3	Primary with 1 bud	Green	
17	6	10,8,8,7,8,5	Primary with 1 bud	Green	
18	7	11,9,9,8,7,7,6	Primary with 2 bud	Green	

Total dry Mass = 0.0763g

Results of Experiment M2R2 Growth on Burgeff's N_3f Medium + Vitamins

Tube #	No. of Pitchers	Length of Pitchers (mm)	Roots	Color	Observations
1	3	4,4,3	Primary	Yellow brown	
2	2	3,3	Primary	Brown	Stopped development approx. week 2
3	2	2,4	Primary	Brown	Stopped development approx. week 2
4	3	4,5,4	Primary	Yellow Brown	
5	5	6,5,3,3,3	Primary	Yellow	
6	3	4,3,3	Primary	Yellow brown	
7	2	3,4	Primary	Brown	
8	3	4,4,3	Primary	Yellow Brown	
9	4	4,4,3,3	Primary	Yellow green	
10	5	5,5,5,4,3	Primary with 1 bud	Yellow	
11	2	3,3	Primary	Yellow brown	
12	1	3	Primary	Brown	Stopped development approx. week 2
13	2	3,3	Primary	Brown	Stopped development approx. week 2
14	3	4,3,3	Primary	Yellow	
15	5	6,6,5,4,4,3	Primary with 1 bud	Yellow green	
16	4	5,4,4,3	Primary	Yellow brown	
17	4	4,4,4,3	Primary	Brown	
18	5	6,5,5,4,4	Primary	Yellow green	

Total dry weight = 0.0187g

Results of Experiment M2R2 Growth on Sphagnum - based Medium + Vitamins

Tube #	No of Pitchers	Length of Pitchers (mm)	Roots	Color	Observations
1	3	4,3,3,	Primary	Yellow green	
2	1	3	Primary	Brown	Stopped development approx. week 1
3	1	3	Primary	Brown	Stopped development approx. week 1
4	3	5,4,3	Primary	Yellow green	
5	4	4,5,5,3	Primary with 1 secondary	Yellow green	
6	5	5,4,4,4,3	Primary with 1 secondary	Yellow green	
7	4	5,4,4,4	Primary with 1 secondary	Yellow green	
8	5	5,5,4,4,3	Primary with 1 secondary	Green	
9	5	6,5,5,4,4	Primary with 1 secondary	Green	
10	5	4,5,5,3,3	Primary with 1 secondary	Green	
11	6	6,6,5,4,4,3	Primary with 1 secondary	Green	
12	4	4,4,3,3	Primary	Yellow green	
13	5	6,4,4,3,3	Primary dichotomously branched	Yellow green	
14	2	3,3	Primary	Brown yellow	Stopped development approx. week 2
15	1	3	Primary	Brown	Stopped development approx. week 1
16	4	5,3,4,3	Primary dichotomously branched	Yellow green	
17	6	5,5,5,4,4,3	Primary dichotomously branched	Green	
18	5	5,4,4,3	Primary dichotomously branched	Yellow green	

Total Dry Mass = 0.0194 g

Results of Experiment Root # 1

Experiment RT # 1 – Treatment # 1 Liquid ½ Strength MS + Vitamins						
	Flask # 1		Flask # 2		Flask # 3	
	Total # of pitchers = 196		Total # of pitchers = 172		Total # of pitchers = 208	
	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 12	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 9	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 13
1	2.1		3.1		2.5	
2	2.5		2.8		2.3	
3	2.4		2.5		2.1	
4	2.0		2.0		2.4	
5	2.6		2.3		2.0	
6	2.3		2.4		2.0	
7	2.5		2.1		2.4	
8	2.4		2.3		2.5	
9	2.0		2.4		2.6	
10	2.8		2.4		2.5	
11	2.7		2.0		2.1	
12	2.5		2.0		2.3	
13	2.3		1.8		2.2	
14	2.4		2.0		2.0	
15	2.5		2.3		2.1	
16	2.6		2.4		2.1	
17	2.1		2.5		2.3	
18	2.3		2.5		2.2	
19	2.4		1.9		2.4	
20	2.4		1.9		2.4	
21	2.0		2.0		2.0	
22	2.1		2.1		2.3	
23	2.3		2.4		2.4	
24	2.5		2.0		2.5	
25	2.1		1.8		2.5	
	Total Dry Mass = 0.5280g		Total Dry Mass = 0.4240g		Total Dry Mass = 0.4915g	

Occasional multiple roots

Results of Experiment Root # 1

Experiment RT # 1 – Treatment # 3 Liquid ¼ Strength MS + Vitamins						
	Flask # 1		Flask # 2		Flask # 3	
	Total # of pitchers = 220		Total # of pitchers = 188		Total # of pitchers = 172	
	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 5	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 4	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 6
1	2.6		2.6		2.8	
2	2.3		2.5		3.0	
3	2.4		2.4		2.1	
4	2.8		2.1		2.5	
5	2.1		2.3		2.6	
6	2.9		2.9		2.4	
7	2.2		3.0		2.3	
8	2.7		2.8		2.0	
9	2.5		3.0		2.1	
10	2.5		2.1		2.0	
11	3.0		2.4		2.5	
12	2.4		2.6		2.4	
13	2.2		2.1		2.6	
14	2.2		2.0		2.5	
15	2.5		2.7		2.5	
16	2.1		2.8		2.8	
17	2.3		2.4		2.3	
18	2.5		2.3		2.1	
19	2.2		2.5		2.6	
20	2.5		2.6		2.5	
21	2.6		2.4		2.4	
22	2.8		2.1		2.0	
23	2.3		2.2		2.0	
24	2.4		2.7		2.1	
25	2.5		2.3		2.6	
	Total Dry Mass = 0.3062g		Total Dry Mass = 0.2701g		Total Dry Mass = 0.2300g	

Multiple roots not present

Results of Experiment Root # 1

Experiment RT # 1 – Treatment # 4 Liquid ¼ Strength MS + Vitamins + Charcoal						
	Flask # 1		Flask # 2		Flask # 3	
	Total # of pitchers = 124		Total # of pitchers = 155		Total # of pitchers = 138	
	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 4	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 3	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17 # of Roots present = 5
1	4.5		4.4		4.0	
2	4.6		4.2		4.6	
3	3.8		3.8		4.5	
4	4.0		3.9		4.1	
5	3.4		3.5		3.4	
6	3.8		3.6		3.5	
7	3.6		3.2		3.0	
8	3.6		2.9		2.9	
9	3.0		2.8		3.2	
10	4.0		3.3		3.3	
11	3.5		3.4		4.0	
12	4.0		3.6		3.3	
13	2.8		3.7		3.4	
14	3.2		3.0		4.0	
15	2.8		3.0		3.2	
16	3.7		4.1		3.2	
17	3.3		3.5		3.0	
18	2.9		3.4		3.4	
19	3.0		3.3		3.4	
20	3.6		3.0		3.5	
21	4.1		2.8		2.8	
22	4.0		2.9		2.9	
23	3.5		3.1		3.7	
24	3.3		3.2		3.5	
25	4.0		3.2		3.1	
	Total Dry Mass = 0.2977g		Total Dry Mass = 0.3381g		Total Dry Mass = 0.3900g	

Multiple roots not present

Results of Experiment Root # 2

Experiment RT # 2 – Treatment # 1 Liquid ½ Strength MS + Vitamins						
	Flask # 1		Flask # 2		Flask # 3	
	Total # of pitchers = 180		Saved for future experiments		Contaminated	
	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17				
1	2.6					
2	2.9					
3	3.0					
4	2.3					
5	2.1					
6	2.4					
7	2.4					
8	2.6					
9	2.1					
10	2.8	# of Roots present = 10				
11	2.8					
12	2.4					
13	2.6					
14	2.5					
15	2.1					
16	2.5					
17	2.0					
18	2.1					
19	2.1					
20	2.4					
21	2.6					
22	2.8					
23	2.1					
24	2.0					
25	2.5					
	Total Dry Mass = 0.488g					

Results of Experiment Root # 2

Experiment RT # 2 – Treatment # 2 Liquid ½ Strength MS + Vitamins + Charcoal						
	Flask # 1		Flask # 2		Flask # 3	
	Total # of pitchers = 152		Saved for future experiments		Saved for future experiments	
	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17				
1	4.3					
2	4.1					
3	3.6					
4	3.7					
5	3.6					
6	3.2					
7	3.0					
8	2.8					
9	3.1					
10	3.4	# of Roots present = 19				
11	3.6					
12	3.8					
13	3.5					
14	3.5					
15	3.5					
16	3.2					
17	3.5					
18	4.0					
19	3.6					
20	3.8					
21	3.0					
22	3.0					
23	3.2					
24	3.4					
25	3.2					
	Total Dry Mass = 0.556g					

Results of Experiment Root # 2

Experiment RT # 2 – Treatment # 3 Liquid ¼ Strength MS + Vitamins						
	Flask # 1		Flask # 2		Flask # 3	
	Total # of pitchers = 233		Saved for future experiments		Contaminated	
	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17				
1	2.8					
2	2.8					
3	2.5					
4	2.5					
5	2.3					
6	2.4					
7	2.2					
8	2.8					
9	2.7					
10	2.5	# of Roots present = 7				
11	2.2					
12	2.2					
13	2.1					
14	3.0					
15	2.3					
16	2.6					
17	2.1					
18	2.7					
19	2.4					
20	2.6					
21	2.2					
22	2.1					
23	2.4					
24	2.4					
25	2.5					
	Total Dry Mass = 0.322g					

Results of Experiment Root # 2

Experiment RT # 2 – Treatment # 4 Liquid ¼ Strength MS + Vitamins + Charcoal						
	Flask # 1		Flask # 2		Flask # 3	
	Total # of pitchers = 134		Contaminated		Contaminated	
	Length of longest 20 pitchers (cm)	Roots # of Rhizome segments examined = 17				
1	4.1					
2	4.3					
3	4.2					
4	3.9					
5	3.9					
6	3.5					
7	3.6					
8	3.4					
9	3.5					
10	4.0	# of Roots present = 6				
11	4.0					
12	4.1					
13	3.4					
14	3.4					
15	3.5					
16	3.0					
17	3.3					
18	3.2					
19	3.6					
20						
21	3.0					
22	3.2					
23	3.2					
24	3.4					
25	3.5					
	Total Dry Mass = 0.2977g					

RESULTS ANCILLARY TO MANUSCRIPT III

Results of Metamorphosis Experiments: Series 1- *Darlingtonia californica*

Experiment # Morph 1A
Start Date 5/18/02

Seedlings approximately 4 weeks old that had germinated in H_20 treatments from DC51 and DC52 were transferred to solid (8.5 g/l agar) POMM (200 ml in 500 ml flasks), 12 seedlings were transferred to each flask. Flasks were placed in the growth chamber. As of 5/21/02 many seedlings were beginning to turn brown in both flasks. As of 7/28/02 three seedlings in flask #1 formed protocorm-like structures and green, leafy callus. Two seedlings in flask #2 formed similar masses of tissue.
Duration of experiment – 70 days

Experiment # Morph 1B (continuation of Morph 1A)
Start Date 7/30/02

The callus and protocorm-like structures formed in experiment 1A were each cut into 3 smaller segments for a total of 15 segments. Five segments were transferred to each of 3, 50 ml Erlenmeyer flasks containing 10 ml of liquid POMM. Flasks were placed in the growth chamber.
As of 8/15/02 (15 days of subculture) the segments in all 3 flasks show growth of shoots from both the segments of protocorm-like bodies and the green, leafy callus.
On 9/14/02 the flasks were photographed (44 days of subculture).

Experiment # Morph 1C (continuation of Morph 1A and 1B)
Start Date 9/15/02

Following 45 days of subculture the clumps of shoots produced in two of the flasks were divided and subcultured again. The remaining flask was sacrificed for drawings and observations. Clumps of plants were evenly distributed among 3, 250 ml Erlenmeyer flasks each containing 75 ml of liquid POMM. The flasks were placed in the growth chamber. During the next 7 days there was an initial dieback of pitcher leaves. This was followed by a spurt of new growth. As of 10/15/ the plants in the flasks appeared healthy and were continuing to multiply.

Experiment # Morph 2
Start Date 7/30/02

Three seedlings, 3 months old, from experiment DC51 that had remained in 50 ml Erlenmeyer flasks containing 10ml of ½ strength liquid MS medium were divided into 2

pieces each (by this time the seedlings had formed dense clusters of pitchers). Two clusters were placed in each of 3, 500 ml Erlenmeyer flasks containing 50 ml of liquid POMM. The flasks were placed in the growth chamber. On 8/15/02 one flask was used for experiment Morph 3B.By 9/17/02 the plants in the remaining flasks had multiplied. One of the remaining 2 flasks was sacrificed to experiment Morph 3C.

Experiment #Morph 3A

Start date – 7/30/02

This experiment was performored to test the regenerative ability of *in vitro* grown pitchers. Plantlets from experiment DC GA1 were used. Pitchers were removed under sterile conditions and cut into 2-3 pieces. Pieces were placed in each of 3, 50 ml Erlenmeyer flasks containing 10 ml of MM medium. The flasks were placed in a growth chamber at 27°C with a 16 hr photoperiod.

Experiment Morph 3A		
Flask # 1	Flask # 2	Flask # 3
6 fragments	7 fragments	7 fragments
Aug. 15 –no new growth	Aug. 15 – 1 well developed plantlet	Aug.15 – 2 fragments showing growth of protocorm like structures
Sept. 14 –no new growth	Sept. 14 – photographed Drawn on Sept. 15	Sept. 14 - photographed Drawn on Sept. 15
3 of a total of 20 fragments formed new growth structures This was the preliminary experiment that led to experiment Morph 2		

Results of Experiment # Morph 3B
Start date – 8/15/02 – end date – 9/15/02

This experiment was performed to test the regenerative ability of whole pitchers, basal halves of pitchers, distal halves, pitcher fragments and whole seedlings. Explant material was placed in 8 ml of liquid POMM in 125 mm X25 mm test tubes and placed in the growth chamber.

Tube #	Treatment #1 Fragmented pitchers 2 mm-5 mm in length	Treatment #2 Whole Pitchers 0.75 cm to 1.0 cm in length - 1 per tube	Treatment #3 Basal portion of pitchers cut in half – 1 per tube	Treatment #4 Distal portion of pitchers cut in half – 1 per tube	Treatment #5 2 whole plantlets from March germination – grown on solid MS – 1 per tube
As of 9/15/02					
1	4 of 8 fragments shoe new growth – others are brown	Brown – no new growth	Brown – no new growth	Brown – no new growth	One shows greening and new growth
2	4 of 8 fragments shoe new growth – others are brown	Brown – no new growth	Brown – no new growth	Brown – no new growth	Turned brown – no new growth present
3	2 fragments of 10 show new growth - others are brown	Brown – no new growth	Brown – no new growth	Brown – no new growth	
4	8 of 8 are brown	Brown – no new growth	Brown – no new growth	Brown – no new growth	
5	8 of 8 are brown	Brown – no new growth	Brown – no new growth	Brown – no new growth	
6	1 fragment of 8 shows new growth – others are brown	Brown – no new growth	Brown – no new growth	Brown – no new growth	
	11 of 50 fragments show new growth	0 of 18 show new growth			1 of 2 shows new growth

Metamorphosis –Results of Exp. # Morph 3C
Start Date 9/17/02

Explant material for this experiment was obtained from a flask from Exp. Morph 2. Explants were placed in 125 mm x 25 mm test tubes containing 10 ml of liquid MM Medium and placed in the growth chamber at 27°C with a 16 hour photoperiod. Pitchers used were all 10 mm-18 mm in length. Data collected 10/17/02

Experiment Morph 3C									
Tube #	Treatment #1 Whole Pitchers	Treatment #2 Basal 1/2	Treatment #3 Distal ½	Treatment #4 Basal Fragments	Treatment #5 Distal Fragments	Treatment #6 Serial Fragments			
						6 a	6 b	6 c	6 d
1	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
4	-	Remained green	Remained green	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-
6	-	-	-	remained green	Remained green	-	-	-	-
7	-	New pitcher formed	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-
15	Growth from tip	-	-	-	-	-	-	-	-
	1 of 15 new growth	1 of 15 new growth	0	0	0	0	0	0	0

- = Browned and died (no new growth)

Experiment # Morph 4
Start Date 9/18/02
End Date 10/15/02
(36 day duration)

2-3 week old seedlings from experiment DCGEM2002C, treatment #2 (24 hr soak in H_2O) and treatment #3 (24h hr in GA_3) were transferred from water agar to corning 25 ml polystyrene tissue culture flasks containing 9 ml of liquid POMM. Four seedlings per flask, four replicates from each treatment.

Results as of 10/15/02

Flask #	Treatment # 1 (seedlings from DCGEM2002C treatment #2)	Treatment #2 (seedlings from DCGEM2002C treatment #3)
1	All 4 brown	3 show very green protocorm-like structures, 1 brown
2	2 show green growth 2 brown	1 green, long pitcher 3 brown
3	2 show green but browning 2 brown	2 very green 1 beginning to brown 1 brown
4	1 shows new green growth of protocorm-like structure 3 brown	2 green 2 brown
	5 of 16 show new growth	8 of 16 show new growth

Experiment # Morph 5
Start Date 9/15/02

This experiment was conducted to gather preliminary observations on growth of *D. californica* in full strength liquid MS. 3-4 week old seedlings from exp. DCGEM2002C, treatment #2 (24 soak in H_2O) were placed, 6 per flask, in 75 ml of liquid full strength MS medium in 250 ml erlenmyer flasks and placed in the growth chamber. As of 10/15/02 1 has browned and the others remain green but growth is very slow. Photographs were taken on 10/16/02.

Results of Metamorphosis Experiments Series 2 – *Sarracenia leucophyll, S. purourea*

Experiment Morph #6A
Start Date – 7/30/02

Two seedlings of S. leucophylla that had germinated on 3/2/02 on MYP (as part of surface sterilization experiments) were transferred on 3/15/02 to solid ½ strength MS medium in 125 mm X25 mm test tubes. On 4/15/02 the two seedlings showed little growth and no multiplication, They were transferred to solid POMM. On 7/30/02 (after 3 1/2 months of growth), during which they produced few new shoots and increased rhizome length only slightly, they were transferred to a 500 ml Erlenmeyer flask containg 50 ml of liquid POMM. BY 9/15/02 they had formed clusters of pitchers with large rhizomes that could be divided, these were divided into several pieces and placed in three, 25 0ml erlenmyer flasks containing 75 ml of liquid POMM and returned to the growth chamber. On 10/20/02 they were photographed, divided and placed in three, 500 ml Erlenmeyer flasks containing 100 ml of POMM.

Experiment Morph # 6B
Start Date – 9/15/02

When 6 month old pitchers from Morph #6A were transferred to new medium, four pitchers were fragmented and seven fragments were placed in each of four plastic tissue culture flasks containing 9 ml of liquid POMM. All were placed in the growth chamber under the same conditions that had allowed regeneration in *D. californica* fragments.
As of 10/15/02, all had turned brown. Two turned slightly red after the first week and appeared to swell but after 30 days they had turned brown and apparently died.

Experiment Morph # 7
Start Date – 4/29/02

Two young pitchers were harvested from mature greenhouse grown plants (1 from each plant). One pitcher was approx. 6-7 weeks old and the other approx. 2-3 weeks old The pitchers were cut from the plants at the base of zone 5 using a sterile dissecting scissors. Care was taken not to include any portion of the rhizome. Following surface sterilization (20% Clorox, 10 min, 3, 3 min rinses) sections were cut from each zone with a sterile scalpel. The sections were placed in 250 ml Erlenmeyer flasks containing 75 ml of POMM. Three flasks were used for each pitcher with the sections being placed in the following order:

	Flask 1	Flask2	Flask 3
2-3 week old pitcher	1 zone 5, 2 zone 4	2 zone 3	1 zone 2, 2 zone1
5-7 week old pitcher	1 zone 5, 2 zone 4	2 zone 3	1 zone 2, 2 zone1
5/6/02 – Flask 1 and 2 contaminated in 5-7 week old pitcher – no contam. In 2-3 wk old 5/12/02 All flasks, all segments, contaminated			

Experiment Morph # 8
Start Date – 8/1/02

Three unopened pitchers were harvested from three individuals in a population in the Great Swamp. Pitcher sizes were 2.8 cm, 4.0 cm and 6.2 cm. Pitchers were washed for 15 minutes in slowly running tapwater + 3 drops of Tween-20, surface sterilized in 3% H_2O_2 for 5 min, then cut into zonal segments. The largest pitcher opened during washing. Zonal segments were cut and placed in liquid POMM (10 ml) in 50 ml Erlenmeyer flasks with five flasks used per pitcher. Segments were placed in flasks as follows:

zone	2.8 cm pitcher # of segments	4.0 cm pitcher # of segments	6.2 cm pitcher # of segments
1	1	1	2
2	1	1	1
3	2	1	1
4	2	2	5
5	1	2	3
As of 8/12 all flasks had contaminated			

Experiment Morph # 9
Start Date – 9/5/02

One, unopened pitcher (1.8 cm in height) was harvested from a greenhouse grown plant, cleaned in a mild detergent for several minutes under running tapwater and surface sterilized in 3% H_2O_2 for 10 minutes. The pitcher was cut into nine fragments, 2 mm in length, in sequence from zone 5 (#1 fragment) to zone 1(#9 fragment) and one fragment per tube was placed in each of 9, 125 mm X 25 mm test tubes containing 10 ml of liquid POMM. The tubes were placed in the growth chamber. After, 24 hr. contamination was observed in tube #1. After 48 hrs contamination was observed in tube 2. Contamination was observed in tubes 3 and 4 after 72 hrs. At the end of seven days all had contaminated.

APPENDIX B

A REVIEW OF SELECTED PAPERS FROM THE LITERATURE ON THE TISSUE CULTURE AND MICROPROPAGATION OF CARNIVOROUS PLANTS

ABSTRACT

Selected papers from the literature regarding micropropagation of carnivorous plants have been reviewed. Current techniques in the literature for the surface disinfestation of seeds and subsequent micropropagation from *in vitro* generated plant material are discussed and tables presented. A baseline series of protocols has been extracted from the literature for the *in vitro* study of carnivorous plants in the laboratory.

INTRODUCTION

Carnivorous plant species have long been objects of study because they possess unique physiological and anatomical adaptations peculiar to the carnivorous habit (see Lloyd, 1942, for early references). This collection of artificially grouped plants comprises over 600 species (Adamec, 1997) and has grown considerably since 1989 when Givnish reported 538 species. The taxonomic occurrence of these plants is spread over 10 families and 19 genera (table1). Givnish reported eight families and 18 genera in 1989. This group is important for several reasons. Several members of the genus *Drosera* and the related single species genus *Dionaea* are used in the preparation of medicinal compounds and for the extraction of other secondary metabolites (Bobak et al., 1995; Bobak et al., 1993; Budzianowski, 2001; 1997; 1996;

1995; Crouch et al., 1990; and Zenk and Steglich, 1969. Collection pressure and/or habitat destruction has reduced or depleted many natural populations of carnivorous plants in the wild (Anthony, 1992). Many species of carnivorous plants are considered threatened or endangered with many currently listed in the list of protected species at either the national or international level by the Convention on International Trade in Endangered Species (Boulay, 1995).

Carnivorous plants are increasingly being used as model systems for the study of fundamental problems in plant development (Bobak et al., 1995; Bobak et al., 1993; Leichtscheidle, et al., 1989; Samaj, et al., 1995). The widespread use of plant tissue culture as a technique for micropropagation has resulted in an increase in recent research on carnivorous plants. Many species such as *Nepenthes, Sarracenia, Darlingtonia, Cephalotus* and the many *Drosera* species are avidly sought by private collectors and possess particular horticultural value. Additionally, Baskin and Baskin (1998) group the carnivorous plant species collectively under the heading of plants with specialized life cycles or habitats.

With the work on the Arabidopsis genome nearing completion it is timely to begin investigating and developing other plant model systems. The carnivorous plants present an opportunity to study a variety of unique nutritional, ecological, morphological, and developmental adaptations which have arisen independently in several plant families and genera that share a common ecology and habit.

The literature contains very little specific information on seeds and embryos of carnivorous plants. It has been summarized by Baskin and Baskin (1998). These authors tabulated dormancy requirements and germination requirements for seeds of

13 carnivorous plant genera. However, except for the single species genera of *Aldrovanda*, *Cephalotus*, *Darlingtonia*, *Dionaea* and *Drosophyllum* their table is not species specific and germination of carnivorous plants in the laboratory under aseptic conditions is not addressed.

The widespread use of plant tissue culture as a technique for micropropagation has resulted in an increase in recent research on carnivorous plants. Much of the literature reports the use of surface sterilized seeds as a source of explant material for *in vitro* culture. Various methods are reported for breaking dormancy and achieving germination. While it is not within the scope of these papers to address problems regarding types of dormancy, clarification of the problem will facilitate future research in developing micropropagation protocols for additional species. One problem that has to be addressed is that the use of disinfectants in the laboratory generally negates data from application to the ecology and timing of germination in nature since seeds in nature are exposed to many types of soil microorganisms and environmental factors (Baskin and Baskin, 1998). However, knowledge of the phenology of the seed phase of the plant life cycle can contribute significantly to successful germination in the laboratory.

Baskin and Baskin (1998) suggest that most species of carnivorous plants have dormant seeds with some being non-dormant. According to these authors, physiological dormancy is the most common type of dormancy found within the carnivorous plant group with a few species having morphological or morphophysiological dormancy.

The following selective, chronology details some of the history of laboratory work with carnivorous plants. Data and observations pertaining to seeds and/or surface sterilization of seeds and other explant tissues has been extracted and presented in a series of tables. In summary, any attempt to establish a micropropagation system or micropropagation protocols for selected carnivorous plant species must begin with a survey of the literature.

A LOOK AT THE LITERATURE

Burgher (1961) - Burgher reported a method for the sterilization of seeds of *Drosera intermedia*, a germination protocol, and a medium that allowed only limited growth of plantlets. Most important were his observations that the highest germination occurred when the seeds were exposed to a cycle of 12 h of light at 38°C followed by 12 h of light at 15°C. No dark period was necessary. However, when seeds were exposed to 12 h of darkness at 15°C, germination was approximately 13% less than that obtained in constant light. Seeds were illuminated with two, 40 watt, cool-white fluorescent bulbs. Germination occurred after five weeks of treatment. Burgher reported that the medium used for his experiments was not satisfactory for continued growth and maintenance of the seedlings. He also reported that exposure of the seeds to IAA (indole-3-acetic acid), gibberellic acid, dilute sulfuric acid, high temperature (37°C) or freezing followed by either high or room temperature did not enhance germination. He did not report concentrations or exposure times.

Pringsheim and Pringsheim (1962) - These researchers obtained axenic cultures of *Uticularia exoleta* in liquid culture using a modification of a medium developed for the growth of the green alga *Micrasterias*. Paramount among their experimental results are the observations that peptone and meat extract added to the basal medium not only promoted superior growth of the plants but also caused the plants to flower. They reported the concentrations employed as 0.05% each of Difco-tryptone and Difco-beef extract. They also reported that peptone, used alone, improved growth slightly but beef extract was necessary for flower production. Additionally, they reported that growth of cultured plants was much improved when the medium was prepared by dissolving iron sulfate crystals after the chelating agent (EDTA) was added to the medium and before addition of other trace elements.

Withner (1964) - Withner was the first to report a protocol for the culture of immature seeds (described by Withner as fertilized ovules) from surface sterilized seed capsules of carnivorous plants. He also reported a medium that allowed sustained growth and proliferation of cultured plantlets of *Darlingtonia californica*, *Dionaea muscipula*, *Sarracenia flava*, and *S. purpurea*. He prepared a germination medium composed of chopped fresh sphagnum (amount not reported), sucrose (not quantified), and potassium nitrate (not quantified). This medium gave excellent germination (not quantified), growth and proliferation. However, he states growth was enhanced by 40% on Burgeff N_3f medium (a medium used for the germination of orchids), plus 0.5 g/l casamino acids (casein hydrolysate). Additionally, he reported that substituting peat moss for sphagnum resulted in similar germination and growth.

However, sphagnum allowed the best percentage of germination and growth. In his experiments, plants grown in the dark on Burgeff N_3f medium were still able to grow enough to fill the flasks. According to Withner, *Darlingtonia* proliferated very rapidly on Burgeff N_3f medium. Lighting conditions were 16 h of fluorescent light followed by 8 h of darkness.

Swamy and Ram (1969) - Surface sterilized seeds of *Utricularia inflexa* were plated on modified White's medium (liquid and semisolid; 0.8% agar). Germinated seedlings were subsequently used as sources of explant material for further *in vitro* studies. Explant material consisted of internodes (0.5-1.5 cm in length), nodes with half each of the two adjacent internodes (0.8 cm in length), portions of the stolons comprising 2-4 nodes (2.0-2.5 cm long), shoot tips (1.0-1.5cm long) and leaf lobes (0.5cm long). All explants were successful except internodal explants which failed to reorganize any meristematic tissue. Also reported was good vegetative growth when 500 mg/l of beef extract, casein hydrolysate, peptone, tryptone, or yeast extract were added to the basal medium. They observed the following effects of growth substances on seed germinating and seedling morphology. 1) GA_3 at 3.4 ppm stimulated germination; 2) GA_3 at 6.8 ppm depressed germination;3) IAA inhibited germinations at both concentrations tested (5 ppm and 10 ppm); 4) kinetin at 0.5 ppm and 1.0 ppm stimulated germination; 5) Neither kinetin nor GA_3 caused any significant change in seedling morphology; 6) IAA at 10 ppm caused etiolation of seedlings, epinasty of cotyledonoids, slender stolons, and more finely dissected leaf lobes than those of the control group.

Chandler and Anderson (1976) - Hydrolytic enzymes associated with the leaves and tentacles of *Drosera* species and their role in heterotrophic nutrition were studied. During the course of these studies *Drosera binata* was introduced into axenic culture. Seeds less than four weeks old were harvested and surface sterilized in a solution of benzalconium chloride (0.1% w/v) and calcium proprionate (18 mM). Sterilization time was 15 min. The seeds were next washed with sterile distilled water and transferred to 500 ml flasks containing 180 ml of culture medium as described by Lidell (1953). However, the agar percentage was reduced to 0.65% (w/v). Neither contamination rates nor germination percentages are given. Germinated seedlings were explanted (one per flask) and raised under a bank of fluorescent and grow-lux tubes. The tubes supplied 8.5 Wm^{-2} at the agar surface. Insects were autoclaved and applied aseptically to the plants as part of the nutritional studies.

Adams et al., (1979a) - These workers reported the clonal multiplication of *Cephalotus follicularis* on ½ strength Linsmaier-Skoog medium with 30 g/l sucrose and 8 g/l agar (pH adjusted to 5.7-5.8 prior to agar addition). Thiamine and inositol were also reduced to ½ strength. Shoot tips were excised, surface sterilized and initially cultured on the above medium with 0.1 mg/l indolebutyric acid (IBA) and 1.0 mg/l benzyladenine (BA). The cultures were kept in the dark for 6 weeks to prevent the accumulation of phenolic oxidation compounds that form in the presence of light and often kill the explanted tissue. The tissue was then transferred for 8 weeks to conditions of constant light (2.2-3.2 klx) and constant temperature (21-25°C).

Following the light and temperature treatment the tissue was transferred to a medium containing 0.1mg/l napthaleneacetic acid (NAA) and 0.1mg/l BA. They reported rapid proliferation of plantlets on this medium with a 5-10 fold increase in plantlet numbers after each subculture. They noted that full strength medium produced less vigorous growth. Additionally, they reported that the micropropagation of *Cephalotus* was also achieved in liquid culture using the same medium without the addition of agar. Rotated liquid cultures broke apart to form many small clumps while shaken cultures proliferated as a solid mass. Growth in liquid cultures was accompanied by an initial die-back of tissue at pH 5.7. However, no die-back occurred in medium adjusted to pH 5.0. Growth at pH 5.0 was also more rapid.

Adams et al., (1979b) – The *in vitro* propagation of the butterwort *Pinguicula moranensis* from excised, surface sterilized leaves of mature plants *on* 1/5 strength Lindsmaier-Skoog medium was reported. The medium also contained 30g/l sucrose and the pH was adjusted to 6.5 before adding 6 g/l agar. Leaves placed on full strength medium died. Leaves cultured on this medium without hormones produced an average of three leaves per leaf. Leaves from these aseptic seedlings grown on the above medium were transferred to the same medium containing hormones (0.02 mg/l 6-benzylamino purine (BA) and 0.01 mg/l napthaleneacetic acid (NAA)). This hormonal ratio produced a combined maximum number of plantlets and growth rate. Plantlet yield was increased by subculturing to higher hormonal levels (BA 2.0 mg/l: NAA 1.0 mg/l) for three to four weeks then plantlets were subcultured back to the lower hormonal concentrations to maximize growth. These workers estimated that a

single leaf producing seven to eight, three leaved plantlets could be expected to produce 500 plantlets in six months of culture.

Beebe (1980) - Beebe described aseptic germination, callus formation, and adventitious bud development in *Dionaea muscipula*. He germinated seeds on full strength MS medium with the following supplements: thiamine, 0.1mg/l; nicotinic acid, 0.5 mg/l; pyridoxine, 0.5 mg/l; glycine, 2 mg/l; myo-inositol, 100 mg/l; casein hydrolysate, 1.0 g/l; sucrose, 20 g/l; coconut milk, 1.5% (v/v); and 1.0% agar. The pH of the medium was adjusted to 5.6 before autoclaving. Beebe reported germination and early seedling development to be as described by Smith (1931) for seeds germinated in soil under greenhouse conditions. He stated that over a four -year period, several different seed lots were used with germination ranging from 78% in a sample of 95 seeds to 40% in a sample of 150 seeds. Seeds germinated throughout a 10-55 day period with the majority (over 70%) germinating between 10 and 35 days.

Various concentrations of growth substances were applied to this medium for developmental studies. The growth substances used were 1-napthaleneacetic acid (NAA) and N6-benzyladenine (BA). High ratios of NAA to BA promoted the production of roots from both aseptically germinated seedlings and from callus tissue. Equal ratios of NAA to BA promoted the proliferation of callus and callus buds from both cultured seedlings and callus other callus buds. He also observed that seedlings on medium containing 10^{-7} M of NAA and 10^{-5} M BA formed large, fleshy adventitious buds at the tips of broad petioles. The petiole margins on these plantlets were ruffled. These buds, which he termed "callus-buds", formed in place of traps at

the leaf tips. Beebe also reported that rapid callus formation and bud growth occurred on media containing 10^{-6} M of NAA + 10^{-7} M of BA, 10^{-6} M of NAA+ 10^{-6} M of BA and 10^{-7} M of NAA + 10^{-6} M of BA. Lastly, he reported that compact, slow growing, green callus was obtained on a medium containing 10^{-8}M NAA + 10^{-5}M of BA.

Parliman et al., (1982a) – Parliman and co-workers used single rhizome explants from aseptically germinated seedlings as a source of explant material to establish proliferating cultures of *Dionaea muscipula* (venus flytrap). Seeds were germinated on MS medium. However the authors are not specific regarding the strength of the medium. Staba vitamins (Staba, 1969), 100 mg/l myo-inositol, 100 mg/l casein hydrolysate, and 30 g/l sucrose were also added to the germination medium. The pH was adjusted to 5.9 before adding agar (6.7 g/l). Following germination, plantlets were subcultured to ½ strength basal MS medium (including the additional components listed above) plus 1.9 mg/l napthaleneacetic acid (NAA) and 0.2 mg/l 6-benzlamino purine (BA). The pH was adjusted to 4.9 prior to the addition of 6.7 g/l agar. Explants grown on this medium showed a 5-14 fold increase in the number of healthy, vigorous, rooted plantlets in 60 days. Plantlets were next transferred to an acclimatization or pre-transplant medium. The authors state that this medium allowed the rapid increase in size of the subcultured plantlets but did not allow an increase in the number of plantlets. The composition of the medium was ½ strength MS medium with organic components as above except the NAA and BA were removed and GA_3 at 0.3 mg/l or 1.0 mg/l was added. The authors reported that after 40 days growth on this medium plantlets were ready to be transferred *ex vitro*.

Parliman et al., (1982b) – Parliman and co-workers reported adventitious bud differentiation and development from tissue cultured leaf cuttings of *Dionaea muscipula*. The source of the leaf cuttings for these experiments was aseptically germinated seedlings as described in their previous work (Parliman et al., (1982a). They expected adventitious buds to arise independently from the leaf tissue. However, secondary buds developed from the rhizomes of primary adventitious buds with each secondary bud initiating another secondary bud in a chain-like fashion. They termed these secondary buds lateral buds (LB) as opposed to the primary adventitious bud (AB). They presumed that these buds developed from meristematic tissue within the AB rhizomes. They also stated that the origin of the AB buds is unknown (whether from single or multiple cell origin). The medium employed for these experiments was ½ strength MS with organic supplements as described earlier (Parliman et al., 1982a). Hormonal concentrations that produced the greatest number of AB and LB buds were NAA at 1.9 mg/l +6(-y-y-dimethylallylamino)-purine (2iP) at 0.2 mg/l. Additionally, they reported that leaves dipped for 24 h in 2iP at 2.1 mg/l produced the greatest number of adventitious and lateral bud derived plantlets when subsequently cultured on the above medium.

Bonnet et al., (1984) - Whole plants and seeds of *Drosera rotundifolia* were field collected near Spa (Belgium Ardennes). Both whole plants and seeds were surface sterilized for 15 min in 2% bleach followed by two washes in sterile distilled water. Seeds were germinated on ¼ strength MS medium (Murashige and Skoog,

1962). The MS medium was supplemented with various concentrations of BAP (benzylamino purine) and NAA (Napthaleneacetic acid). The range of concentrations was not reported. The authors reported 10% seed germination on ¼ strength MS alone and 28% when the seeds were treated with GA (gibberellic acid). No concentration or time is given for the GA treatment. Seeds were also given a 30-day cold pretreatment at 4° C on the MS medium. Cold treated seeds gave 90% germination at room temperature without treatment with GA.

Culture of *in vitro* explants from mature plants was not successful because of ineffective surface sterilization or death of the tissue. Contamination rates of surface decontaminated seeds and explant tissues are not given.

Further assays were completed using sterile leaves from *in vitro* germinated seedlings. Length of culture times was not given. Excised leaves subcultured on ¼ strength MS alone produced a few adventitious buds. The authors reported that these buds formed on both the lamina and petiole segments of the explanted tissue. The number of buds increased when the medium was supplemented with BAP or NAA but the concentrations leading to the increase are not reported. However, they did report that more than 20 buds per explant were obtained using 10^{-5} M BAP with or without NAA. Only roots were formed in the presence of 10^{-5} M NAA and low concentrations of BAP; no time spans were given for the appearance of the buds. Finally, the authors reported that in most of the hormonal combinations the newly formed buds initiated roots a few days after their first appearance. While no histological photographs or drawings are shown, they reported that roots were well

connected with the shoots and complete plantlets were able to develop on the ¼ strength MS medium alone.

Bobak et al., (1989) -Histological studies of organogenesis in the leaves of *Drosera spathulata* L. *in vitro* were presented. Seeds were greenhouse harvested and surface sterilized in 2.5% chloramine for 15 min followed by several washes with sterile distilled water. The seeds were germinated in petri dishes containing MS medium and charcoal. The strength of the MS medium and the amount of charcoal added were not reported. Seeds were cold treated at 4° C for 30 days before cultivation. It is not clear if this cold treatment preceded or followed surface sterilization and plating on the MS medium. Following 80 days of cultivation, sterile leaves were removed from the plants and placed on a modified medium (MS, 1962) with added grapevine exudates (5%). The authors do not give any references for the preparation of the exudate. Leaves were sampled periodically at 6, 9, 15 and 21 days for developing organoids (this term is taken to mean new shoot meristematic regions). Tissue was prepared for both TEM and light microscopy. They reported that visual observations of the tissue on day six revealed no apparent changes. However, serial sections of the tissue revealed that cell division had already begun to take place in specific regions at an earlier time.

They reported the formation of large clusters of dividing cells located in the inner portions of the explant tissue that may be associated with, or lying close to, the vascular system. These cells are described as being highly vacuolate with a well-defined nucleus. They also describe cells entering into the division process that are

highly reminiscent of cells found in shoot apical meristems. That is, they apparently have a high affinity for cytological stains and show an increased accumulation of starch. These centers of division are reported to arise as a proliferation of parenchymatous tissue and may include the cells of the subepidermal layer. The authors state that by day 21 these regions have given rise to protuberances that upon histological examination possess distinctly differentiated shoot meristematic apices and leaf primordial. With continued culture, complete plants were regenerated that were able to live under normal conditions (normal conditions are not defined).

MICROPROPAGATION OF *NEPENTHES KHASIANA*

Reference:

Rathore, T.S., Tandon, P. & Shekhawat, N.S. (1991). *In vitro* regeneration of pitcher plant (*Nepenthes khasiana* Hook. f.) – a rare insectivorous plant of India. Journal of Plant Physiology, 139, 246-248.

In Vitro Germination of Seeds

Surface sterilization protocol:

1) Wash several times with tap water and a few drops of Tween 80.
2) Surface sterilize with 0.1% (w/v) mercuric chloride for 2-3 minutes.
3) Wash with sterile H_2O.
4) Soak in sterile water for 3-4 hours.
5) Submerge in 70% ethanol for 20 seconds.
6) Rinse with sterile water.
7) Inoculate on germination medium.

Germination medium:

1) ½ strength Murashige and Skoog (1962)
2) 0.1 MG/L IAA
3) 0.5 mg/l kinetin
4) 2.0 mg/l GA3
5) 20 g/l sucrose
6) Adjust pH of medium to 5.8 before adding agar.
7) 8.0 g/l agar
8) Store cultures under diffuse light at 26+/- 2 degrees Celsius.
9) Use 6-8 week-old seedlings far nodal and apical shoot explants (1.0-1.5 cm explant length).
10) Transfer explants to multiplication medium.

Multiplication medium:

1) Full strength MS medium
2) 0.1 mg/l IAA
3) 2.0 mg/l BAP
4) 50.0 mg/l ascorbic acid
5) 25.0 mg/l citric acid
6) 50.0 mg/l arginine
7) 25.0 mg/l adenine sulfate
8) 500.0 mg/l charcoal
9) 500.0 mg/l polyvinylpyrrolidone

10) Adjust pH of medium to 5.8 before adding agar.
11) 8.0 g/l agar
12) Keep cultures in the dark for 3-4 days.
13) Transfer to light conditions of 2500 lux light intensity for 12 hours photoperiod at 26 ± 2°C.
14) After 4 weeks growth, differentiated shoots may be subcultured for further multiplication or transplanted to rooting medium.

Rooting Medium:

1) ½ strength MS medium
2) 2.0 mg/l NAA
3) 0.1 mg/l kinetin
4) 20 g/l sucrose
5) Adjust pH to 5.8 before adding agar.
6) 8.0 g/l agar
7) Store cultures in the dark for 1 week.
8) Transfer to light conditions of 2500 lux light intensity at 26 +- 2 degrees Celsius.
9) Transfer to strengthening medium for faster growth and development of pitchers.
10) Approximately 80% of the plantlets should develop roots.

Strengthening and pitcher development medium:

1) ¼ strength MS medium (hormone-free)
2) 20 g/l sucrose
3) Adjust pH of medium to 5.8 prior to addition of agar.
4) 6 g/l agar
5) Light and temperature conditions as above.
6) When plantlets are approximately 5-7 inches in length with well developed roots, remove from flasks, wash thoroughly with tap water followed by sterile distilled water, and then transfer to pots containing vermiculite and drained soil (3:1)
7) Harden in a growth chamber.
8) Within 4-5 weeks, potted plants should develop pitchers with normal lids.

Additional Notes

Nodal shoot segments were found to produce the most multiple shoots (10-12). Apical shoot explants produced only 6-8 new shoots. Ascorbic acid, citric acid, arginine and adenine sulfate enhanced shoot production (12-15 per explant) when added to the multiplication medium. If they were not added the cultures declined in growth and gradually deteriorated. Leaf and root explants did not produce shoots or callus. Hardening before transplantation to a growing medium was essential.

MICROPROPAGATION OF *DROSERA ROTUNDIFOLIA* – PRODUCTION OF ADVENTITIOUS BUDS

Reference:

Bobak, M., Blehova, A., Kristin, J., Ovecka, M., & Samaj, J. (1995). Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured *in vitro*. Plant Cell, Tissue and Organ Culture, 43, 43-49.

Explant source:

Leaves from aseptically grown plantlets of *D. rotundifolia*. The authors neither state nor reference a protocol for the production of the aseptic plantlets. However, see Anthony (1992) and Burgher (1961). The authors report that the source plants were grown on MS medium (Murashige and Skoog, 1962) with a 16-hour photoperiod (PPF of 32uM/m2/s) under cool white fluorescent bulbs at a temperature of 24 ± 2°C.

Medium for the induction of adventitious buds:

The authors reported testing 49 different media formulations and report best production occurred on liquid MS medium with 10-8M of NAA (18.4 plantlets per explant). Solidified MS medium without growth regulators produced approximately 7 adventitious buds per leaf explant.

1) Full strength MS medium (basal salts)
2) 30 g/l sucrose
3) 100 mg/l myo-inositol
4) Adjust the pH to 5.8 before adding agar or use liquid medium (for liquid induction medium add 10-8M of NAA).
5) 7 g/l agar

Medium for the induction of callus:

1) Full strength MS medium and supplements as above for solid medium plus10^{-6} or $5x10^{-6}$ M of BA
2) Place in light and temperature conditions as outlined under Protocol.

Protocol for the generation of adventitious buds:

1) Transfer leaves excised from aseptically cultured plants to either solidified MS medium with no growth regulators (abaxial side in contact with the medium) or to liquid MS medium supplemented with 10^{-8} M of NAA.
2) Place liquid medium cultures on a gyratory shaker at 120 rpm.

3) Maintain both liquid and solid media with a 16-hour photoperiod under cool white fluorescent lights with a PPF of 32 umol/m^2/s (400-700 nm).
4) Maintain solid and/or liquid cultures at 24 ± 2°C.

Additional Notes

A change in the leaf pigmentation from green to dark red or red to dark red occurred two days after culture initiation. Media supplemented with 0 to 2 x 10^{-5} M NAA induced intensive red pigment formation with some necrosis after 28 days in culture. Optimal callus formation and proliferation occurred in the light only on medium containing 10^{-6} or 5x 10^{-6} M BA. The callus formed was light green and very compact. Full strength, solidified MS medium without hormones produced the most direct shoot formation of all solidified formulations tested. Buds were observed to form on the whole leaf surface, but in particular near the tentacles.

MICROPROPAGATION OF *DROSERA SPATHULATA*
SHOOT TIP CULTURE
INDUCTION OF *IN VITRO* FLOWERING

Reference:

Perica, M.C & Berljak, J. (1996). *In vitro* growth and regeneration of *Drosera spathulata* Labill. on various media. HortScience, 31, 1033-1034.

Initial explant material: - surface disinfested shoot tips.

Protocol for surface sterilization of shoot tips:

1) Excise shoot tips 4-6 mm in length from greenhouse-cultivated plants (>2.5 cm in diameter).
2) Cut off leaves and excise shoot apices.
3) Surface-sterilize excised shoot apices in 1.0% sodium hypochlorite (SH) plus one drop of Tween 20 surfactant for 5 minutes (higher concentrations of SH caused explants to turn brown and decay, as did ETOH dips).
4) Rinse three times (3 minutes each) in sterile distilled water.
5) Inoculate separately on full-strength MS medium (Murashige and Skoog, 1962) in glass culture tubes containing 10 ml of medium.
6) Maintain cultures with a 16-hour photoperiod under cool white fluorescent lights (40umol m^2/s) and at a temperature of 24 ± 2°C .
7) Subculture after 3 months (each tube should contain 100-200 plantlets).

Medium preparation:

1) Full-strength MS medium including vitamins
2) 30 g/l sucrose
3) Adjust pH to 5.7 with NaOH before autoclaving.
4) 8.0 g/l agar

Subculture:

The authors state that it was not necessary to use the usual 3-stage propagation system for shoot induction, multiplication and rooting. They observed that medium without a growth regulator was best for regeneration and multiplication. Plants rooted spontaneously on this medium. Leaves from regenerated shoots may also be subcultured to fresh medium for adventitious bud induction and subsequent development of shoots. However, regeneration occurred 2 weeks later than when whole plants were subcultured.

Induction of flowering:

Plants can be induced to flower by subculturing to ¼ strength MS medium supplemented with 0.12uM IBA plus 0.44 μM BA. This medium reportedly induced 100% flowering. Plants subcultured from ¼ strength MS medium at pH 5.7 to ¼ strength MS medium at pH 4.0 obtained the largest diameter (in 8 weeks subsequent growth) in comparison to plants subcultured to ¼ strength media at higher pH values.

MICROPROPAGATION OF *DROSERA ROTUNDIFOLIA*, *D. CAPENSIS* AND *D. BINATA* ADVENTITIOUS PLANTLET FORMATION FROM EXPLANTED WHOLE LEAVES

Reference:

Anthony, L.J. (1992). *In vitro* propagation of *Drosera* spp. HortScience, 27, 850.

Source of explant material: whole leaves excised from greenhouse-cultivated plants.

Surface sterilization of leaves:

Whole leaves were excised and surface disinfested in 10% (v/v) Clorox (0.5% sodium hypochlorite) with 1 drop of Tween 20 surfactant added to each 50 ml of 10% Clorox solution. Excised leaves were disinfested for 5 minutes followed by three 5-minute rinses in sterile distilled water. Longer time spans (10-20 minutes) in the disinfestations solution caused extensive tissue damage and lack of regeneration of adventitious plantlets. Anthony states that approximately 75% of the original cultures contaminated with 95% of the *D. rotundifolia* cultures were lost to contamination. She suggests that the contamination was due to the presence of symbiotic bacteria and fungi that may play a role in the digestive process (see Chandler and Anderson, 1977).

Medium:

1) ½ strength MS medium (Murashige and Skoog, 1962) prepared by diluting full strength MS medium containing vitamins.
2) 30 g/l sucrose
3) 0,02 mg/l BA
4) 0.01 mg/l NAA

Note: Addition of phytohormones may not be necessary for the induction of adventitious plantlets since Anthony states that on both ½ strength MS medium without hormones and ½ strength MS medium supplemented with phytohormones, the multiple adventitious plantlets entirely covered the leaf surface. However, hormones were required for the induction of flowering in *D. binata*.

5) Adjust pH to 5.7 before adding agar.
6) 8 g/l agar

Protocol:

1) Excise whole leaf from plants.

2) Surface-disinfest in 10% (v/v) Clorox plus one drop of Tween 80 surfactant per 50 ml of sterilant solution. Surface sterilize for 5 minutes.
3) Rinse 3 times (5 minutes each) with sterile distilled water.
4) Transfer to 25 x 150 ml culture tubes containing 20 ml of medium.
5) Maintain cultures at 25 °C with a 16-hour photoperiod under fluorescent lights (Philips 40-W Agro-Lite bulbs).
6) Shoots should appear after approximately 4 weeks.
7) For rooting of *D rotundifolia* and *D. capensis*, subculture adventitious shoots to ½ strength MS medium without hormones (Anthony reported the formation of extensive root systems after 6-8 weeks).
8) For rooting of *D. binata*, subculture to ½ strength MS medium with hormones as above. Anthony observed that only 10% of the *D. binata* rooted on medium supplemented with BA and NAA.

Induction of flowering:

Anthony found that about ½ of the regenerated plantlets of *D. rotundifolia* and *D. capensis* produced flowers when subcultured to fresh ½ strength MS medium with or without hormones. She observed that approximately 90% of the *D. binata* plantlets flowered when subcultured to ½ strength MS medium containing hormones. She did not vary hormonal concentrations. *D. binata* did not produce flowers on media without hormones.

ASEPTIC GERMINATION AND GROWTH OF *DROSERA BINATA*

References:

Chandler, G.E. & Anderson, J.W. (1967). Studies on the origin of some hydrolytic enzymes associated with the leaves and tentacles of *Drosera* species and their role in heterotrophic nutrition. New Phytologist, 77, 51-62.

Liddell, R. (1953). Germination of *Paphiopediulum*. American Orchid Society Bulletin 22:195

Surface sterilization of seeds:

1) Disinfest seeds (seeds four weeks old were used in these experiments) for 15 minutes in a solution of benzalconium chloride (0.1% w/v) and calcium propionate (18 mM).
2) Rinse in sterile distilled water.
3) Transfer to sterile 500 ml flasks containing 180 ml of nutrient medium (Chandler and Anderson used Liddell's orchid germination medium and reported lowering the agar concentration to 0.65% w/v).
4) Light and temperature conditions were not stated.
5) Following germination (time required not reported), transfer to fresh medium. For purposes of their experiments Chandler and Anderson transferred 1 seedling per 500 ml flask.
6) For growth, maintain cultures at 20 °C under fluorescent and Gro-lux tubes (8.5 Wm-2 at the agar surface). Photoperiod not reported.

Liddell's orchid germination medium:

$Ca(NO_3)2{\cdot}4H_2O$	1.00 g/l
NH_4NO_3	0.25 g/l
$MgSO_4{\cdot}7H_2O$	0.25 g/l
KH_2PO_4	0.20 g/l
K_2HPO_4	0.10 g/l

To each liter of medium add 5ml of the following stock solution:
6.0 g citric acid. $1H_2O$
1.25 g $FeSO_4.7H_2O$
0.25 $MNSO_4.H_2O$
500 ml distilled water

Liddell also added 20 g of sugar consisting of 90% glucose and 10% sucrose per liter. The pH was adjusted to 5.0. Agar concentration was 1.75%.

IN VITRO CULTURE OF EMBRYOS OF *DROSOPHYLLUM LUSITANICUM*

Reference:

Dore, J., Swamy, R., & Mohan Ram, H.Y. (1967). Cultivation of embryos of *Drosophyllum lusitanicum* Link, an insectivorous plant. Experimentia, 19, 363-371.

Note: This is a very early paper in the history of carnivorous plant tissue culture. However, it contains information regarding techniques, media formulations and experimental results that may be useful in designing future tissue culture experiments.

Outline of experiments:

1) Seeds were surface sterilized with chlorine water (specifics not reported), embryos were excised under aseptic conditions and planted on various media.
2) The media employed were:

Basal medium = modified White's medium + 2% sucrose + 0.8% agar.

Medium #1 = basal medium

Medium #2 = basal medium + casein hydrolysate

Medium #3 = basal medium + casein hydrolysate + IAA (1ppm)

Medium #4 = basal medium + casein hydrolysate + coconut milk (15% v/v)

Medium #5 = basal medium + kinetin (1ppm)

Medium #6 = basal medium + yeast extract (500mg/l)

3) Embryos cultured on Medium #1, Medium #2, Medium #3 and Medium #6 germinated and produced seedlings after 6-8 weeks of culture. The roots were well developed and the plantlets attained a height of 7-8 cm in 10 weeks. Flowering was not observed.
4) Embryos cultured on Medium #5 formed highly condensed, succulent shoots. Leaf bases were fleshy and adhered to the stem. No roots developed.
5) Embryos cultured on Medium #4 did not germinate, but formed proliferating dark brown, friable callus after 10 weeks of culture. Patches of pearly white tissue appeared on these brown calli.
6) The white tissue was excised from these calli and subcultured on Medium #4 and basal medium + casein hydrolysate + kinetin (1 ppm) + IAA (1 ppm). Active cell division occurred in both media.
7) Approximately 12% of the subcultures on Medium #4 differentiated into shoots and roots after two weeks of subculture.

White's medium:

Macronutrients = KNO_3 (80 mg/l); $MgSO_4 \cdot 7H_2O$ (737 mg/l); $NaH_2PO_4 \cdot H_2O$ (19 mg/l); $Ca(NO_3)2 \cdot 4H_2O$ (288 mg/l); KCl (65 mg/l); $NaSO_4$ (200 mg/l).

Micronutrients = Kl (0.75 mg/l); H_3BO_3 (1.5 mg/l); $MnSO_4 \cdot 4H_2O$ (6.65 mg/l); $ZnSO_4 \cdot 7H_2O$ (2.67 mg/l); MoO_3 (0.0001 mg/l); $CuSO_4 \cdot 5H_2O$ (0.001 mg/l); $Fe(SO_4)3$ (2.5 mg/l).

The above formulation of White's medium is from Handbook of Plant Cell Culture Vol. 1. Edited by D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yamada. Macmillan Publishing Company. Copyright 1983. See also Singh, M. and Krikorian, A.D. (1981). White's Standard Nutrient Solution. Annals of Botany 47: 133-139.

MICROPROPAGATION OF *PINGUICULA MORANENSIS*

References:

Adams II, R.M., Koenigsberg, S.S., & Langhans, R.W. (1979b). *In vitro* propagation of the butterwort *Pinguicula moranensis* h.b.k. HortScience, 14, 701-702.

Linsmaier, E. & Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum, 185, 100-127.

Source of explant: whole leaves.

Micropropagation Protocol

Surface sterilization of explants:

1) Excise whole leaves from stock plants.
2) Shake for five minutes in 0.1% Tween 20 surfactant with 3 changes of the solution during this time span (vacuum aspirate and replace with fresh solution).
3) Replace solution with 1% Physan 20 (10% n-alkyl dimethyl benzyl ammonium chloride + 10% n-alkyl dimethyl ethylbenzyl ammonium chloride) and shake for 5 minutes with 3 changes of solution.
4) Replace solution with 10% Clorox (0.525% sodium hypochlorite) with 0.1% Tween 20 surfactant. Shake for five minutes with changes of the sterilant solution.
5) Rinse three times with sterile distilled water.

Note: Succulent winter leaves survive disinfestations much better than summer leaves. Both leaf forms give rise to adventitious plantlets equally well.

Aseptic culture:

1) Transfer surface disinfested leaves to culture tubes or flasks containing Linsmaier-Skoog (1965) medium with all salts, thiamine and inositol at 1/5 strength, 0.02 mg/l BA, 0.01 mg/l NAA, 30 g/l sucrose.
2) Adjust pH of medium to 6.5 before adding agar.
3) 6 g/l agar
4) Leaves cultured on this medium should produce 7-8 plantlets with roots on each leaf explant in approximately 8 weeks.

Subculture:

1) For a higher yield of plantlets, subculture the leaves to 1/5 strength Linsmaier-Skoog medium containing 2.0 mg/l BA and 1.0 mg/l NAA for 3-4 weeks. This results in additional adventitious bud formation.
2) To maximize the growth of each plantlet, subculture again on 1/5 strength Linsmaier-Skoog medium with a reduced hormone level (0.02 mg/l BA and 0.01 mg/l NAA).
3) Acclimatize the plantlets by planting in limed, sterilized sphagnum moss. Gradually reduce the relative humidity from 100% to 50% over a period of 4 weeks.

Note: Linsmaier-Skoog medium contains the same macronutrient and micronutrient salts as Murashige and Skoog (1962) medium and differs only in its vitamin composition. Linsmaier and Skoog increased the level of thiamine four-fold from the original MS formulation. Nicotinic acid and pyridoxine were found unnecessary and eliminated from the composition of vitamin mixture.

Table No.1 Taxonomic Classification of Carnivorous Plants

Kingdom - Planate
Division – Magnoliophyta
Class – Chloripetalae

Order	Family	Genus	*No. of Species
Sarraceniales	Sarraceniaceae	*Darlingtonia*	1
		Heliamphora	6
		Sarracenia	9
Nepenthales	Droseraceae	*Drosera*	90-100+
		Drosophyllum	1
		Dionaea	1
		Aldrovanda	1
	Nepenthaceae	*Nepenthes*	70
Saxifragales	Cephalotaceae	*Cephalotus*	1
	Byblidaceae	*Byblis*	2
Violales	Dioncophyllaceae	*Triphyophyllum*	1

Division – Magnoliophyta
Class – Sympetalae

Order	Family	Genus	*No. of Species
Bromeliales	Bromeliaceae	*Brocchinia*	2
		Catopsis	1
Scrophulariales	Lentibulariaceae	*Pinguicula*	35
		Utricularia	280
		Genlesia	35
		Polyphompholyx	2
		Biovularia	1
	Martynaceae	*Ibicella*	1

* Approximate number of species (from Givnish, 1989; Cheers, 1992).

Genus	Species	Explant Tissue	Comments	Reported by
Drosera	*pygmaea*			Harder (1964)
	natelensis	Mature leaves, young unopened shoots, flower buds, flower stalks, and roots from field collected plants	Report embryogenesis in all explant sources except the root which they were unable to decontaminate	Crouch and van Staden (1987)
Drosera	*rotundifolia*	leaves from aseptically grown plants	Report embryogenic callus	Samaj et al. (1995)
	rotundifolia	Aseptic seedlings		Bonnet et al. (1984)
	rotundifolia	Aseptic seedlings		Simola (1978a)
	rotundifolia	Aseptic seedlings produced in Simola experiments (1978a)		Simola (1978b)
	rotundifolia	Leaves from aseptically grown plants		Bobak et al. (1995)
	rotundifolia			Kulczanka and Cznsika (1987)
	rotundifolia	Whole leaves		Anthony (1992)
	spathulata	Leaves from aseptically grown plants		Bobak et al. (1989)

Genus	Species	Explant Tissue	Comments	Reported by
Drosera	*pygmaea*			Harder (1964)
	natelensis	Mature leaves, young unopened shoots, flower buds, flower stalks, and roots from field collected plants	Report embryogenesis in all explant sources except the root which they were unable to decontaminate	Crouch and van Staden (1987)
Drosera	*rotundifolia*	leaves from aseptically grown plants	Report embryogenic callus	Samaj et al. (1995)
	rotundifolia	Aseptic seedlings		Bonnet et al. (1984)
	rotundifolia	Aseptic seedlings		Simola (1978a)
	rotundifolia	Aseptic seedlings produced in Simola experiments (1978a)		Simola (1978b)
	rotundifolia	Leaves from aseptically grown plants		Bobak et al. (1995)
	rotundifolia			Kulczanka and Cznsika (1987)
	rotundifolia	Whole leaves		Anthony (1992)
	spathulata	Leaves from aseptically grown plants		Bobak et al. (1989)

Genus	Species	Explant Tissue	Comments	Reported by
Drosera	*spathulata*	Leaves from aseptically grown plants	Report callogenesis	Blehova et al. (1992)
	spathulata	Shoot tips		Perica and Berljak (1996)
	spathulata	Leaves from aseptically grown plants	Studied plastid morphogenesis	Bobak et al. (1996)
	spathulata	Leaves from aseptically grown plants	Report callus induction	Bobak et al. (1993)
Drosophyllum	*lusitanicum*	Seeds, excised embryos	Report of embryo culture	Swamy and Ram (1967)
Genlesia				not reported
Heliamphora				not reported
Ibicella				not reported
Nepenthes	*khasiana*	Aseptic seedlings, leaf tissue, nodal cuttings, shoot apices, and root segments		Rathore et al. (1991)
	½ of known species of *Nepenthes*			Redwood and Bowling (1990) Kew Gardens
Pinguicula	*moranensis*	Whole leaves		Adams et al. (1979a)
	lusitanica			Harder and Zemlis (1967)
Polyphompholyx				not reported
Sarracenia	*purpurea*	Immature seed		Withner (1964)
	flava	Immature seed		Withner (1964)

Genus	Species	Explant Tissue	Comments	Reported by
Triphyophylum	*flava*	Seeds, axenic seedlings		Bringmann and Rischer (2001)
Utricularia	*exoleta*	Nodal segments		Pringsheim and Pringsheim (1962)
	inflexa	Stolon segments		Swamy and Ram (1971)
	inflexa	Ripe fruits		Swamy and Ram (1969)

Literature Cited

Adamec, L. (1997). Mineral nutrition of carnivorous plants: A review. The Botanical Review, 63, 273-299.

Adams II, R.M., Koenigsberg, S.S., & Langhans, R.W. (1979a). *In vitro* propagation of *Cephalotus follicularis* (Australian pitcher plant). HortScience, 14, 512-513.

Adams II, R.M., Koenigsberg, S.S., & Langhans, R.W. (1979b). *In vitro* propagation of the butterwort *Pinguicula moranensis* h.b.k. HortScience, 14, 701-702.

Anthony, L.J. (1992). *In vitro* propagation of *Drosera* spp. HortScience, 27, 850.

Beebe, J.D. (1980). Morphogenetic responses of seedlings and adventitious buds of the carnivorous plant *Dionaea muscipula* in aseptic culture. Botanical Gazette, 141, 396-400.

Baskin, C.C. & Baskin, J.M. (1998). Seeds; ecology, biogeography, and evolution of dormancy and germination. San Diego, CA. Academic Press.

Beeckman, T., De Rycke, R., Viane, R., & Inze, D. (2000). Histological study of seed coat development in *Arabidopsis thaliana*. Journal of Plant Research, 113, 139-148.

Bewley, J.D. & Black, M. (1994). Seeds – Physiology, Development and Germination (2nd ed.). New York, London. Plenum Press.

Blehova, A., Erdelsky, K., & Bobak, M. (1992). Cultivation of organ and callus culture of *Drosera spathulata* Labill. in *in vitro* conditions. Physiologia Plantarum 27, 93-101.

Bobak, M., Blehova, A., & Erdelsky, K. (1989). Histological and cytological study of early phases of organogenesis on excised leaves of *Drosera spathulata* L. cultivated *in vitro*. Biologia, 44, 795-792.

Bobak, M., Blehova, A., Cholvadova, B., & Somsakova, V. (1990). Morphogenesis of the plastid apparatus in the process of organogenesis at the leaves of the sundew (*Drosera spathulata* L.), cultivated in *in vitro* conditions. Physiologia Plantarum, 25, 33-41.

Bobak, M., Blehova, A., Kristin, J., Ovecka, M., & Samaj, J. (1995). Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured *in vitro*. Plant Cell, Tissue and Organ Culture, 43, 43-49.

Bobak, M., Blehova, A., Samaj, J., & Ovecka, M. (1993). Studies of organogenesis from the callus culture of the sundew (*Drosera spathulata* Labill.). Plant Physiology, 142, 251-253.

Bobak, M., Samaj, J., Blehova, A., Ovecka, M., Novomeska, S., & Kristin, J. (1998). Morphology and ultrastructure of isolated gemmae of *Drosera pygmaea* and their *in vitro* germination. Biologia Plantarum, 41, 169-176.

Boulay, J. (1995). Les plantes carnivores, essais de micropropagation. Bulletin des Academie et Societe Lorraines des Sciences, 34, 151-159.

Budzianowski, J. (1995). Naphthoquinones of *Drosera spathulata* from *in vitro* cultures. Phytochemistry, 40, 1145-1148.

Budzianowski, J. (1996). Naphthohydroquinone glucosides of *Drosera rotundifolia* and *D. intermedia* from *in vitro* cultures. Phytochemistry, 4, 1145-1147.

Budzianowski, J. (1997). 2-methylnaphthazarin 5-O-glucoside from the methanol extracts of *in vitro* cultures of *Drosera* species. Phytochemistry, 44, 75-77.

Budzianowski, J. (2000). Naphthoquinone glucosides of *Drosera gigantea* from *in vitro* cultures. Planta Medica, 66, 667-669.

Burgher, R.M.(1961). Axenic seedlings of *Drosera*. *Nature*, 191, 724-725.

Chandler, G.E. & Anderson, J.W. (1967). Studies on the origin of some hydrolytic enzymes associated with the leaves and tentacles of *Drosera* species and their role in heterotrophic nutrition. New Phytologist, 77, 51-62.

Cheers, G. (1992). A Guide to Carnivorous Plants of the World. New York. Angus & Robertson, Harper Collins Publishers.

Crouch, I.J., Finnie, J.F., & van Staden, J. (1990.) Studies on the isolation of plumbagin from *in vitro* and *in vivo* grown *Drosera* species. Plant Cell, Tissue and Organ Culture, 21, 79-82.

Crouch, I.J. & van Staden, J. (1987). *In vitro* propogation of *Drosera natalensis*. South African Journal of Botany, 54, 94-95.

Dore, J., Swamy, R., & Mohan Ram, H.Y. (1967). Cultivation of embryos of Drosophyllum lusitanicum Link, an insectivorous plant. Experimentia, 19, 363-371.

Dore, J., Swamy, R., & Mohan Ram, H.Y. (1969). Studies on growth and flowering in axenic cultures of insectivorous plants. I. Seed germination and establishment of *utricularia inflexa*. Phytomorphology, 19, 363-371.

Dore, J., Swamy, R., & Mohan Ram, H.Y. (1971). Studies on the growth and flowering in axenic cultures of insectivorous plants. II. Induction of flowering and development of flower in *Utricularia inflexa*. Zeitschrift fur Pflanzenphysiologie, 65, 315-325.

Givnish, T.J. (1989). Ecology and evolution of carnivorous plants. In W. G. Abrahamson. Plant-Animal Interactions. (pp. 243-290). New York. McGraw-Hill Inc.

Harder, R. (1964). Vegetative entwicklung und blutenbildung in axenischen kulturen der insektivore *Drosera pygmaea* Dc. Planta, 63, 316-325.

Harder, R. & Zemlin, I. (1967). Forderung der entwicklung und des bluhens von *Pinguicula lusitanica* durch futterung in axenischer kultur. Planta (Berl.), 73, 181-193.

Lichtscheidl, I.K., Lancelle, S.A., & Hepler, P.K. (1990). Actin-endoplasmic reticulum complexes in *Drosera*. Protoplasma, 155, 116-126.

Liddell, R. (1953). Germination of *Paphiopediulum*. American Orchid Society Bulletin 22:195

Linsmaier, E. & Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum, 185, 100-127.

Lloyd, E.F. (1942). The Carnivorous Plants. Waltham, Massachusetts. Chronica Botanica Co.

Miles, D.H., Kokpol, U., Zalkow, L.H., Steindel, S.J., & Nabors, J.B. (1974). Preliminary investigation of antitumor activity of *Sarracenia flava*. Journal of Pharmaceutical Science, 63, 613.

Minocha, S.C. (1985.) *In vitro* propagation of *Dionaea muscipula*. HortScience, 20, 216-217.

Munday, R. & Munday, C.M. (2000). Induction of quinone reductase and glutathione transferase in rat tissues by juglone and plumbagin. Planta Medica, 66, 399-402.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15, 473-497.

Parliman, B.J., Evans, P.T., & Mazur, A.R. (1982). Adventitious bud differentiation and development in leaf cuttings of *Dionaea muscipula* Ellis ex. l. (venus fly trap) cultured *in vitro*. Journal of the American Society of Horticultural Science, 107, 310-316.

Parliman, B.J., Evans, P.T., & Rupert, E.A. (1982). Tissue culture of single rhizome explants of *Dionaea muscipula* Ellis ex. l., the venus fly trap, for rapid asexual propagation. Journal of the American Society of Horticultural Science, 107, 305-310.

Perica, M.C & Berljak, J. (1996). *In vitro* growth and regeneration of *Drosera spathulata* Labill. on various media. HortScience, 31, 1033-1034.

Pringsheim, E.G. & Pringsheim, O. (1962). Axenic culture of *Utricularia*. American Journal of Botany, 49, 898-901.

Rathore, T.S., Tandon, P. & Shekhawat, N.S. (1991). *In vitro* regeneration of pitcher plant (*Nepenthes khasiana* Hook. f.) – a rare insectivorous plant of India. Journal of Plant Physiology, 139, 246-248.

Redwood, G. & Bowling, D. (1990). Micropropagation of *Nepenthes* species. Botanic Gardens Micropropagation News, 1, 19-20.

Samaj, J., Bobak, M., Blehova, A., Kristin, J., & Auxtova-Samajova, O. (1995). Developmental SEM observations on an extracellular matrix in embryogenic calli of *Drosera rotundifolia* and *Zea mays*. Protoplasma, 186, 45-49.

Sarma, K.S., Maesato, K., Hara, T., & Sonoda, Y. (1990). Effect of method of agar addition on post-autoclave pH of the tissue culture media. Annals of Botany, 65, 37-40.

Schnell, D.E. (2002). Carnivorous Plants of the United States and Canada (2nd ed.). Portland, Oregon. Timber Press.

Simola, L.K. (1978). Dipeptides as nitrogen sources for *Drosera rotundifolia* in aseptic culture. Physiology of Plants, 44, 315-318.

Smith, C.M. (1931). Development of *Dionaea muscipula* II. Germination of seed and development of seed to maturity. Botanical Gazette, 91, 377-394.

Withner, C.L. (1964). Notes on the cultivation of insectivorous plants *in vitro*. Bulletin of the Torrey Botanical Club, 91, 412-413.

Zenk, M.H., Furbringer, M., & Steglich, W. (1969). Occurrence and distribution of 7-methyljuglone and plumbagin in the *Droseraeceae*. Phytochemistry, 8, 2199-2200.

APPENDIX C

Adventitious or Not?

This appendix contains some thoughts on the formation of adventitious structures in plants.

Traditionally, and for convenience, plant anatomists and morphologists have considered the primary plant body to be composed of three organs: roots, stems and leaves. The stem, leaves and attendant structures are generally referred to as the shoot system. These organs are seen to arise with a predictable, positional regularity and modularity along the root/shoot axis. In other words, the essential entity in development is the dynamic event. The expression of such an event is the pattern (Harrison, 1992). Any organ that arises outside of this basic developmental model or event sequence/pattern in an abnormal position is termed "adventitious". Gray (1887) termed aerial roots of orchids to be "anomalous" structures. Other early literature makes reference to "morphological roots" to describe roots developing from tissues other than the pericycle of the main root (Ames and McDaniels, 1947). Fahn (1974) describes adventitious structures in plants as "an organ that developed in an unusual position". Raven, Evert and Eichorn (1993) describe the term adventitious as "referring to a structure arising from an unusual place, such as buds at other places than leaf axis, or roots growing from stems and leaves". Whether or not any structure is truly adventitious in nature (that is, arising from an unusual place, and not part of the dynamics of a deeper developmental level of pattern expression) is a fundamental question in plant biology.

The phenomenon of adventitious development in vascular plants also belongs to that class of morphogenic events usually referred to as regeneration in the classical botanical literature. These phenomena include, but are not limited to, the formation of adventitious roots, shoots, plantlets and somatic embryos.

Regeneration in plants has long been known and studied. It has been broadly defined as the growth of new plant parts after the removal of corresponding parts elsewhere in the plant (Dore, 1955, includes references to early studies and reviews). In view of current biochemical and molecular techniques, and physiological and anatomical interpretations, this definition needs amendment to include organogenesis on the plant body as well as organogenesis, histogenesis and somatic embryogenesis in tissue surgically excised from the plant and grown aseptically *in vitro*.

Therefore, it is suggested that the term "regeneration" be taken to include any cell division and subsequent differentiation sequence that results in the formation of a complete tissue, organ, plantlet, or somatic embryo or embryo-like structure within either the plant body or tissues excised from the plant and maintained in culture. Such excised tissues are generally termed "explants" (Arditti, 1993).

A confusing number of descriptive terms have accumulated in the literature regarding all aspects of adventitious development. For instance Goebel (1903), in an early review, considered the phenomena of regeneration to imply a development of dormant or latent rudiments, which he termed 'Anlagen'. These Anlagen were already present in the plant, either as dormant, differentiated embryonic tissue - and thus distinguishable either histologically or cytologically from surrounding tissue - or as a tendency or disposition in the tissue toward the formation of new structures. In this latter case, Goebel describes this

tissue disposition as being outwardly invisible. Ames and McDaniels (1947), in their text on plant anatomy, state that adventitious roots may arise from meristematic root germs or 'cushions' that are cytologically or histologically distinguishable from the surrounding cells; or, in contrast, from groups of cells that are not distinguishable from the surrounding tissue, though they are still capable of forming new structures. These are additional terms for Goebel's 'Anlagen' and 'tissue disposition'. Esau (1967) states that adventitious roots may arise from dormant primordia that had been laid in place earlier in development until stimulated to grow, or from primordia that arise anew in apparently undifferentiated tissue.

Sinnott (1960) in Plant Morphogenesis included a chapter concerning regeneration in higher plants in which he divides regenerative phenomena into three different regenerative activities: reconstitution, which applies to cases of reorganization of embryonic tissue in which the original structure is reformed after having been disrupted (regeneration proper); restoration, whereby missing tissue or organs are replaced by the activity of new meristematic regions arising in adjacent tissues (i.e., the formation of new vascular tissues following wounding); and reproductive regeneration, which includes the formation of a new plant or plants from tissues or organs separated from the plant body. Sinnott states that in restoration the missing tissues or organs are replaced by the activation of dormant buds or primordia already present in the tissue, or from the formation of new buds. These primordia, and the formation of primordia *de novo* are another reference to Goebel's 'Anlagen' and 'tissue disposition'.

Current views of morphogenesis in plants employ new terminology to describe the events that occur in the activation of pre-positioned primordia and the initiation of *de novo*

centers of regeneration. Recent advances in understanding cell-to-cell communication and control of differentiation at the biochemical, molecular and physical levels of organization have led to the synthesis of developmental ideas that, when applied to regeneration, necessitate a regrouping of these phenomena under the broader heading of developmental theory. Steeves and Sussex (1989, pg. 336) remark that it is important to recognize that, in *de novo* regeneration it is not an organized root, shoot or flower that is being initiated but rather a meristem. This meristem may not be determined until sometime after its initiation and prior to its gaining autonomous control over its developmental fate. Thus, the entire realm of adventitious development can be seen as the study of pre-formed (and in some cases, still indeterminate) meristematic centers or primordia, or the study of, what appears to be, the *de novo* formation of meristematic zones (Anlagen?). In each case, the cells or tissues involved may be seen as being at the center of a morphogenic field, with the meristem being a morphogenic unit and its activities establishing the boundaries of a larger field of developmental influence.

The ideas discussed above were originally written to serve as an introduction to a study of *de novo* meristem formation with particular emphasis on the role of the epidermis as a potent generative and formative tissue system. Interest in this area is founded on a desire to understand plants in terms of organismal theory. A return to this work is anticipated in the future.

Literature Cited

Arditti, J. (1982). Orchid seed germination and seedling culture – a manual. In J. Arditti (ed), Orchid Biology – Reviews and Perspectives II. Ithaca, New York. Comstock Publishing Associates, Cornell University Press.

Dore, J. (1955). Studies in the regeneration of horseradish. 1. A re-examination of the morphology and anatomy of regeneration. Annals of Botany, 19, 127-137.

Eames, A. J. and Mac Daniels, L.H. (1947). An Introduction to Plant Anatomy. New York. McGraw-Hill Book Company, Inc.

Esau, K. (1967). Plant Anatomy (2nd ed.). New York. John Wiley and Sons, Inc. Pg.513

Fahn, A. (1974). Plant Anatomy (2nd ed.). Oxford, UK. Pergamon Press.

Goebel, K. (1903). Regeneration in plants. Bulletin of the Torrey Botanical Club, 3(4), 197-205.

Gray, A. (1887). The Elements of Botany for Beginners and Schools (revised ed.). Boston. American Book Company.

Harrison, L. G. (1992). Reaction diffusion theory and intracellular differentiation. International Journal of Plant Science, 153, S76-S85.

Raven, P.H., Evert, R.F., & Eichhorn, S.E. (1992). Biology of Plants (2nd ed.). New York, Worth Publishers. Pg. 449

Sinnot. W. E. (1960). Plant Morphogenesis. New York. McGraw Hill Book Company.

Steeves, T.A. & Sussex, I.M. (1989). Patterns in Plant Development. New York, New York. Cambridge University Press.

Bibliography

Adamec, L. (2002). Leaf absorption of mineral nutrients in carnivorous plants stimulates root nutrient uptake. New Phytologist, 155, 89-100.

Adamec, L. (1997). Mineral nutrition of carnivorous plants: A review. The Botanical Review, 63, 273-299.

Adams II, R.M., Koenigsberg, S.S., & Langhans, R.W. (1979a). *In vitro* propagation of *Cephalotus follicularis* (Australian pitcher plant). HortScience, 14, 512-513.

Adams II, R.M., Koenigsberg, S.S., & Langhans, R.W. (1979b). *In vitro* propagation of the butterwort *Pinguicula moranensis* h.b.k. HortScience, 14, 701-702.

Albert, V.A., Williams, S.E., & Chase, M.W. (1992). Carnivorous plants: phylogeny and structural evolution. Science, 257, 1491-1495.

Amen, R.D. (1967). The effects of gibberellic acid and scarification on the seed dormancy and germination in *Luzula spicata*. Physiologia Plantarum, 20, 6-12.

Ames, O. (1899). An easy method of propagating *Drosera filiformis*. Rhodora, 1, 172.

Anthony, L.J. (1992). *In vitro* propagation of *Drosera* spp. HortScience, 27, 850.

Arditti, J. (1982). Orchid seed germination and seedling culture – a manual. In J. Arditti (ed), Orchid Biology – Reviews and Perspectives II. Ithaca, New York. Comstock Publishing Associates, Cornell University Press.

Ball, E. (1956). Growth of the embryo of *Ginkgo biloba* under experimental conditions. I. Origin of the first root of the seedling *in vitro*. American Journal of Botany, 43, 488-495.

Ball, E. (1956). Growth of the embryo of *Ginkgo biloba* under experimental conditions. II. Effects of a longitudinal split in the tip of the hypocotyl. American Journal of Botany, 43, 802-810.

Ball, E. (1959). Growth of the embryo of *Ginkgo biloba* under experimental conditions. III. Growth rates of root and shoot upon media absorbed through the cotyledons. American Journal of Botany, 46 (2), 130-139.

Barrett-Lennard, E.G. & Dracup, M. (1988). A porous agar medium for improving the growth of plants under sterile conditions. Plant and Soil, 108, 294-298.

Barton, L.V. (1965). Viability of seeds of *Theobroma cacao* l. Contributions from Boyce Thompson Institute, 23, 109-122.

Baskin, C.C. & Baskin, J.M. (1998). Seeds; ecology, biogeography, and evolution of dormancy and germination. San Diego, CA. Academic Press.

Beebe, J.D. (1980). Morphogenetic responses of seedlings and adventitious buds of the carnivorous plant *Dionaea muscipula* in aseptic culture. Botanical Gazette, 141, 396-400.

Beeckman, T., De Rycke, R., Viane, R., & Inze, D. (2000). Histological study of seed coat development in *Arabidopsis thaliana*. Journal of Plant Research, 113, 139-148.

Bewley, J.D. & Black, M. (1994). Seeds – Physiology, Development and Germination (2nd ed.). New York, London. Plenum Press.

Bhojwani, S.S. & Johri, B.M. (1971). Morphogenetic studies on cultured mature endosperm of *Croton bonplandianum*. New Phytologist 76, 761-766.

Blehova, A., Erdelsky, K., & Bobak, M. (1992). Cultivation of organ and callus culture of *Drosera spathulata* Labill. in *in vitro* conditions. Physiologia Plantarum 27, 93-101.

Bobak, M., Blehova, A., & Erdelsky, K. (1989). Histological and cytological study of early phases of organogenesis on excised leaves of *Drosera spathulata* L. cultivated *in vitro*. Biologia, 44, 795-792.

Bobak, M., Blehova, A., Cholvadova, B., & Somsakova, V. (1990). Morphogenesis of the plastid apparatus in the process of organogenesis at the leaves of the sundew (*Drosera spathulata* L.), cultivated in *in vitro* conditions. Physiologia Plantarum, 25, 33-41.

Bobak, M., Blehova, A., Kristin, J., Ovecka, M., & Samaj, J. (1995). Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured *in vitro*. Plant Cell, Tissue and Organ Culture, 43, 43-49.

Bobak, M., Blehova, A., Samaj, J., & Ovecka, M. (1993). Studies of organogenesis from the callus culture of the sundew (*Drosera spathulata* Labill.). Plant Physiology, 142, 251-253.

Bobak, M., Samaj, J., Blehova, A., Ovecka, M., Novomeska, S., & Kristin, J. (1998). Morphology and ultrastructure of isolated gemmae of *Drosera pygmaea* and their *in vitro* germination. Biologia Plantarum, 41, 169-176.

Boulay, J. (1995). Les plantes carnivores, essais de micropropagation. Bulletin des Academie et Societe Lorraines des Sciences, 34, 151-159.

Brigham, L.A., Michaels, P.J., & Flores, H.E. (1999). Cell-specific production and antimicrobial activity of naphthoquinones in roots of *Lithospermum erithrorhizon*. Plant Physiology, 119, 417-428.

Budzianowski, J. (2000). Naphthoquinone glucosides of *Drosera gigantea* from *in vitro* cultures. Planta Medica, 66, 667-669.

Budzianowski, J. (1997). 2-methylnaphthazarin 5-O-glucoside from the methanol extracts of *in vitro* cultures of *Drosera* species. Phytochemistry, 44, 75-77.

Budzianowski, J. (1996). Naphthohydroquinone glucosides of *Drosera rotundifolia* and *D. intermedia* from *in vitro* cultures. Phytochemistry, 4, 1145-1147.

Budzianowski, J. (1995). Naphthoquinones of *Drosera spathulata* from *in vitro* cultures. Phytochemistry, 40, 1145-1148.

Burgher, R.M.(1961). Axenic seedlings of *Drosera*. Nature, 191, 724-725.

Busse, J.S. & Evert, R.F. (1999). Pattern of differentiation of the first vascular elements in the embryo and seedling of *Arabidopsis thaliana*. International Journal of Plant Science, 160, 1-13.

Carroll, G. (1988). Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. Ecology, 69, 2-9.

Chandler, G.E. & Anderson, J.W. (1967). Studies on the origin of some hydrolytic enzymes associated with the leaves and tentacles of *Drosera* species and their role in heterotrophic nutrition. New Phytologist, 77, 51-62.

Cheers, G. (1992). A Guide to Carnivorous Plants of the World. New York. Angus & Robertson, Harper Collins Publishers.

Chen, L., James, S.H., & Stace, H.M. (1997). Self-incompatibility, seed abortion and clonality in the breeding systems of several western Australian *Drosera* species (Droseraceae). Australian Journal of Botany, 45, 191-201.

Clay, K. (1988). Fungal endophytes of grasses: a defensive mutualism between plants and fungi. Ecology, 69, 10-16.

Clay, K (1989). Clavicipitaceous endophytes of grasses: their potential as biocontrol agents. Mycological Research, 92, 1-12.

Conran, J.G. & Dowd, J.M. (1993). The phylogenetic relationships of *Byblis* and *Roridula* (Byblidaceae-Roridulaceae) inferred from partial 18S ribosomal RNA sequences. Plant Systematics and Evolution, 188, 73-86.

Crouch, I.J., Finnie, J.F., & van Staden, J. (1990.) Studies on the isolation of plumbagin from *in vitro* and *in vivo* grown *Drosera* species. Plant Cell, Tissue and Organ Culture, 21, 79-82.

Crouch, I.J. & van Staden, J. (1987). *In vitro* propogation of *Drosera natalensis*. South African Journal of Botany, 54, 94-95.

D'Amato, P. (1998). The Savage Garden. Berkeley, California. Ten Speed Press.

Darwin, C. (1875). Insectivorous Plants. London. John Murray.

Das, S., Mitra, S.K., & Mukherjee, K.K. (1995). SEM studies on seed coat of some species of *Ipomoea* (Convolvulaceae). Phytomorphology, 45, 113-125.

Dodds, J.H. & Roberts, L.W. (1995). Experiments in Plant Tissue Culture (3rd ed.). Cambridge. Cambridge University Press.

Dore, J. (1955). Studies in the regeneration of horseradish. 1. A re-examination of the morphology and anatomy of regeneration. Annals of Botany, 19, 127-137.

Dore, J. & Williams, W.T. (1956). Studies in the regeneration of horseradish. 2. Correlation phenomena. Annals of Botany, 20, 231-249.

Dore, J., Swamy, R., & Mohan Ram, H.Y. (1969). Studies on growth and flowering in axenic cultures of insectivorous plants. I. Seed germination and establishment of *Utricularia inflexa*. Phytomorphology, 19, 363-371.

Dore, J., Swamy, R., & Mohan Ram, H.Y. (1971). Studies on the growth and flowering in axenic cultures of insectivorous plants. II. Induction of flowering and development of flower in *Utricularia inflexa*. Zeitschrift fur Pflanzenphysiologie, 65, 315-325.

Eames, A. J. and Mac Daniels, L.H. (1947). An Introduction to Plant Anatomy. New York. McGraw-Hill Book Company, Inc.

Esau, K. (1967). Plant Anatomy (2nd ed.). New York. John Wiley and Sons, Inc. Pg. 513.

Ellison, A.M. (2001). Interspecific and intraspecific variation in seed size and germination requirements of *Sarracenia* (Sarraciniaceae). American Journal of Botany, 88, 429-437.

Ellison, A.M. and Gotelli, N.J. (2001). Evolutionary ecology of carnivorous plants. Trends in Ecology and Evolution, 6, 623-629.

Ellison, A.M. and Parker, J.N. (2002). Seed dispersal and seedling establishment of *Sarracenia purpurea* (Sarraceniaceae). American Journal of Botany, 89, 1024-1026.

Fahn, A. (1974). Plant Anatomy (2^{nd} ed.). Oxford, UK. Pergamon Press.

Franck, D.H. (1975). Early histogenesis of the adult leaves of *Darlingtonia californica* (Sarraceniaceae) and its bearing on the nature of epiascidiate foliar appendages. American Journal of Botany, 62, 116-132.

Franck, D.H. (1976). Comparative morphology and early leaf histogenesis of adult and juvenile leaves of *Darlingtonia californica* and their bearing on the concept of heterophylly. Botanical Gazette, 137, 20-34.

Francko, D.A. (1986). Studies on *Nelumbo lutea* (Wild.) pers. I. Techniques for axenic liquid seed culture. Aquatic Botany, 26, 113-117.

Gallardo, K., Job, C., Groot, S.P.C., Puype, M., Demol, H., Vandekerckhove, J., & Job, D. (2001). Proteomic analysis of *Arabidopsis* seed germination and priming. Plant Physiology, 126, 835-848.

George, E.F. & Sherrington, P.D. (1984). Plant Propagation by Tissue Culture. Edington, UK. Exergetics Limited.

Givnish, T.J. (1989). Ecology and evolution of carnivorous plants. In W. G. Abrahamson. Plant-Animal Interactions. (pp. 243-290). New York. McGraw-Hill Inc.

Godt, M.J.W. & Hamrick, J.L. (1996). Genetic structure of two endangered pitcher plants, *Sarracenia jonseii* and *S. oreophila* (Sarraceniaceae). American Journal of Botany, 83, 1016-1023.

Godt, M.J.W & Hamrick, J.L. (1998). Allozyme diversity in the endangered pitcher plant *Sarracenia rubra* ssp. *alabamensis* (Sarraceniaceae) and its close relative *S. rubra* ssp. *rubra*. American Journal of Botany, 85, 802-810.

Goebel, K. (1903). Regeneration in plants. Bulletin of the Torrey Botanical Club, 3(4), 197-205.

Gotsch, S.G. & Ellison, A.M. (1998). Seed germination of the northern pitcher plant, *Sarracenia purpurea*. Northeastern Naturalist, 5, 175-182.

Gray. A. (1887). The Elements of Botany for Beginners and Schools (revised ed.). Boston. American Book Company.

Harder, R. (1964). Vegetative entwicklung und blutenbildung in axenischen kulturen der insektivore *Drosera pygmaea* Dc. Planta, 63, 316-325.

Harder, R. & Zemlin, I. (1967). Forderung der entwicklung und des bluhens von *Pinguicula lusitanica* durch futterung in axenischer kultur. Planta (Berl.), 73, 181-193.

Harrison. L. G. (1992). Reaction diffusion theory and intracellular differentiation. International Journal of Plant Science, 153, S76-S85.

Hartman, H.T. & Kester, D.E. (1975). Plant Propagation: Principles and Practices (3rd ed.). Englewood Cliffs, New Jersey. Prentice Hall.

Hartman, H.T., Kester, D.E., Davies, Jr., F.T., & Geneve, R.L. (1997). Plant Propagation: Principles and Practice (6th ed.). Englewood Cliffs, New Jersey. Prentice Hall.

Hinton, D.M. & Bacon, C.W. (1984). The distribution and ultrastructure of the endophyte of toxic tall fescue. Canadian Journal of Botany, 63, 36-42.

Hook, I.L.I. (2001). Naphthoquinone contents of *in vitro* cultured plants and cell suspensions of *Dionaea muscipula* and *Drosera* species. Plant Cell, Tissue and Organ Culture, 67, 281-285.

Hu, C.Y. & Wang, P.J. (1983). Meristem, shoot tip and bud cultures. In D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yasuyuki. Handbook of Plant Cell Culture. New York. Macmillan Publishing Co. Pgs. 177-227.

Jasik, J., Salajova, T., & Salaj, J. (1995). Developmental anatomy and ultrastructure in European black pine (*Pinus nigra* Arn.). Protoplasma, 185, 205-211.

Jaffe, K., Michelangeli, F., Gonzalez, J.M., Miras, B., & Ruiz, M.C. (1992). Carnivory in pitcher plants of the genus *Heliamphora* (Sarraciniaceae). New Phytologist, 122, 733-744.

Kane, M.E., Sheehan, T.J., & Ferwerda, F.H. (1988). *In vitro* growth of American lotus embryos. HortScience, 23, 611-613.

Knudson, L. (1946). A nutrient solution for the germination of orchid seed. American Orchid Society Bulletin, 14, 214-217.

Kohlenbeck, H.W. (1978). Comparative Somatic Morphogenesis. In T. A. Thorpe. Frontiers of Plant Tissue Culture. Bet Dagan, Israel. International Association for Plant Tissue Culture.

Koske, R.E. (1977). Spiral growth of *Phycomyces*: some new observations in the Northern and Southern hemispheres. Mycologia, 69, 189-193.

Lackie, S. & Yeung, E.C. (1995). Zygotic embryo development in *Daucus carota*. Canadian Journal of Botany, 74, 990-998.

Lane, W.D. (1979). The influence of growth regulators on root and shoot initiation from flax meristem tips and hypocotyls *in vitro*. Physiologia Plantarum, 45, 260-264.

Lecoufle, M. (1990). Carnivorous Plants: Care and Cultivation. London, England. Cassell Publishers Ltd.

Liddell, R. (1953). Germination of *Paphiopediulum*. American Orchid Society Bulletin 22:195

Leifert, C. & Waites, W.M. (1992). Bacterial growth in plant tissue culture media. Journal of Applied Bacteriology, 72, 460-466.

Leifert, C., Waites, W.M., Camotta, H., & Nicholas, J.R. (1989). *Lactobacillus plantarum*: a deleterious contaminant of plant tissue cultures. Journal of Applied Bacteriology, 67, 363-370.

Leifert, C., Waites, W.M., & Nicholas, J.R. (1989). Bacterial contaminants of micropropagated plant cultures. Journal of Applied Bacteriology, 67, 353-361.

Leifert, C., Waites, W.M., Nicholas, J.R. & Keetley, J.W.W. (1990). Yeast contaminants of micropropagated plant cultures. Journal of Applied Bacteriology, 69, 471-476.

Levitt, R.G. (1903). Reversionary stages experimentally induced in *Drosera intermedia*. Rhodora, 5, 265-272.

Li, X., Baskin, J.M., & Baskin, C.C. (1999). Comparative morphology and physiology of fruit and seed development in the two shrubs *Rhus aromatica* and *R. glabra* (Anacardiaceae). American Journal of Botany, 86, 1217-1225.

Lichtscheidl, I.K., Lancelle, S.A., & Hepler, P.K. (1990). Actin-endoplasmic reticulum complexes in *Drosera*. Protoplasma, 155, 116-126.

Linsmaier, E. & Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum, 185, 100-127.

Lloyd, E.F. (1942). The Carnivorous Plants. Waltham, Massachusetts. Chronica Botanica Co.

Lyshede, O.B. (1984). Seed structure and germination in *Cuscuta pedicellata* with some notes on *C. campestris*. Nordic Journal of Botany, 4, 669-67.

Lyshede, O.B. (1992). Studies on mature seeds of *Cuscuta pedicellata* and *C. campestris* by electron microscopy. Annals of Botany, 69, 365-371.

MacDougal, D.T. (1903). The influence of light and darkness upon growth and development. Memoirs of the New York Botanical Garden, 2, 1-319.

Malgorzata, D.G. (2001). Direct somatic embryogenesis as a rapid and efficient system for *in vitro* regeneration of *Arabidopsis thaliana*. Plant Cell, Tissue and Organ Culture, 64, 39-46.

Malik, K.A. & Saxena, P.K. (1991). Regeneration in *Phaseolus vulgaris* L.: High frequency induction of direct shoot formation in intact seedlings by N^6-benzylaminopurine and thidiazuron. Planta, 186, 384-389.

Mandossian, A.J. (1966). Germination of seeds in *Sarracenia purpurea* (pitcher plant). Michigan Botanist, 5, 67-69.

Martin, A.C. (1946). The comparative internal morphology of seeds. The American Midland Naturalist, 36, 513-589.

Mayer, A.M. & Poljakoff-Mayber, A. (1982). The Germination of Seeds. (3^{rd} ed.). Oxford, UK. Pergamon Press.

McCarty, D.R. (1995). Genetic control and integration of maturation of germination pathways in seed development. Annual Review of Plant Physiology and Plant Molecular Biology, 45, 71-93.

Miles, D.H., Kokpol, U., Zalkow, L.H., Steindel, S.J., & Nabors, J.B. (1974). Preliminary investigation of antitumor activity of *Sarracenia flava*. Journal of Pharmaceutical Science, 63, 613.

Minocha, S.C. (1985.) *In vitro* propagation of *Dionaea muscipula*. HortScience, 20, 216-217.

Morel, G. (1974). Clonal Multiplication of Orchids. In: The Orchids, Scientific Studies. Withner, C.L. (Ed) Wiley-Interscience, New York, Pgs. 169-222.

Munday, R. & Munday, C.M. (2000). Induction of quinone reductase and glutathione transferase in rat tissues by juglone and plumbagin. Planta Medica, 66, 399-402.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15, 473-497.

Parliman, B.J., Evans, P.T., & Mazur, A.R. (1982). Adventitious bud differentiation and development in leaf cuttings of *Dionaea muscipula* Ellis ex. L. (venus fly trap) cultured *in vitro*. Journal of the American Society of Horticultural Science, 107, 310-316.

Parliman, B.J., Evans, P.T., & Rupert, E.A. (1982). Tissue culture of single rhizome explants of *Dionaea muscipula* Ellis ex. l., the venus fly trap, for rapid asexual propagation. Journal of the American Society of Horticultural Science, 107, 305-310.

Pate, J.S. & Dixon, K.W. (1978). Phenology and reproductive biology of the tuberous sundew, *Drosera erythrorhiza* Lindl. Australian Journal of Botany, 26, 441-454.

Perica, M.C & Berljak, J. (1996). *In vitro* growth and regeneration of *Drosera spathulata* Labill. on various media. HortScience, 31, 1033-1034.

Pietropaolo, J. & Pietropaolo, P. (1986). Carnivorous Plants of the World. Portland, Oregon. Timber Press.

Poole, R.J. (1941). Flowers and Flowering Plants (2nd ed.). New York, McGraw Hill Book Co. Inc.

Popham, R.A. (1959). Effects of a low temperature on germination of cotton seeds and on growth of the embryo. The Texas Journal of Science, 11, 299-313.

Pringsheim, E.G. & Pringsheim, O. (1962). Axenic culture of *Utricularia*. American Journal of Botany, 49, 898-901.

Rasmussen, H.N. & Whigham, D.F. (1998). Importance of woody debris in seed germination of *Tipularia discolor* (Orchidaceae). American Journal of Botany, 85, 829-834.

Rathore, T.S., Tandon, P. & Shekhawat, N.S. (1991). *In vitro* regeneration of pitcher plant (*Nepenthes khasiana* Hook. f.) – a rare insectivorous plant of India. Journal of Plant Physiology, 139, 246-248.

Raven, P.H., Evert, R.F., & Eichhorn, S.E. (1992). Biology of Plants (2nd ed.). New York, Worth Publishers. Pg. 449.

Redwood, G. & Bowling, D. (1990). Micropropagation of *Nepenthes* species. Botanic Gardens Micropropagation News, 1, 19-20.

Reeves, R.D., Macfarlane, R.M., & Brooks, R.R. (1983). Accumulation of nickel and zinc by western North American genera containing serpentine-tolerant species. American Journal of Botany, 70, 1297-1303.

Reiser, L., Sanchez-Baracaldo, P., & Hake, S. (2000). Knots in the family tree: evolutionary relationships and functions of *knox* homeobox genes. Plant Molecular Biology, 42, 51-166.

Remeshree, A.B., Hariharan, M., & Unnikrishnan, K. (1994). Micropropagation and callus induction of *Aristolochia bracteolata* Lam. – a medicinal plant. Phytomorphology, 44, 247-252.

Samaj, J., Bobak, M., Blehova, A., Kristin, J., & Auxtova-Samajova, O. (1995). Developmental SEM observations on an extracellular matrix in embryogenic calli of *Drosera rotundifolia* and *Zea mays*. Protoplasma, 186, 45-49.

Sarma, K.S., Maesato, K., Hara, T., & Sonoda, Y. (1990). Effect of method of agar addition on post-autoclave pH of the tissue culture media. Annals of Botany, 65, 37-40.

Schnell, D.E. (2002). Carnivorous Plants of the United States and Canada (2nd ed.). Portland, Oregon. Timber Press.

Schwarz, O.J., & Beaty, R.M. (1996). In R. N. Trigiano & D. J. Gray. Plant Tissue Culture Concepts and LaboratoryExercises. Boca Raton, Florida. CRC Press, Inc.

Sharpe, W.R., Sondahl, M.R., Caldas, L.S., & Maraffa, S.B. (1980). The physiology of *in vitro* asexual embryogenesis. Horticultural Reviews, 2, 268-310.

Shreve, F. (1906). The development and anatomy of *Sarracenia purpurea*. Botanical Gazette, 42, 107-126.

Simola, L.K. (1978). Dipeptides as nitrogen sources for *Drosera rotundifolia* in aseptic culture. Physiologia Plantarum, 44, 315-318.

Sinnot, W. E. (1960). Plant Morphogenesis. New York. McGraw Hill Book Company.

Slack, A. (2000). Carnivorous Plants (3rd impression). Cambridge, Massachusetts. The MIT Press.

Smith, C.M. (1929). Development of *Dionaea muscipula* I. Flower and seed. Botanical Gazette, 87, 507-530.

Smith, C.M. (1931). Development of *Dionaea muscipula* II. Germination of seed and development of seed to maturity. Botanical Gazette, 91, 377-394.

Steeves, T.A. & Sussex, I.M. (1989). Patterns in Plant Development. New York, New York. Cambridge University Press.

Steyermark, J.A. (1984). Flora of the Venezuelan guayana I. Annals of the Missouri Botanical Garden, 71, 297-340.

Sutter, E. & Langhans, R.W. (1979). Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. Journal of American Horticultural Science, 104, 493-496.

Sweet, H.C. & Bolton, W.E. (1979). The surface decontamination of seeds to produce axenic seedlings. American Journal of Botany, 66, 692-698.

Teo, W.L., Kumar, P., Goh, C., & Swarup, S. (2001). The expression of *Brostm*, a *KNOTTED1*-like gene, marks the cell type and timing of *in vitro* shoot induction in *Brassica oleracea*. Plant Molecular Biology, 46, 567-580.

Thorpe, T.A. & Palel, K.R. (1984). Clonal Propagation: Adventitious Buds. In I.K. Vasil (ed.). Cell Culture and Somatic Cell Genetics of Plants (vol. I). Oxford, United Kingdom. Academic Press Inc. Pgs. 49-60

Thurston, K.C., Spencer, S.J., & Arditti, J. (1979). Phytotoxicity of fungicides and bactericides in orchid culture media. American Journal of Botany, 66 (7), 825-835.

Toogood, A. (1999). Plant Propagation. New York, New York. DK Publishing, Inc.

Tran Thanh Van, K. & Trinh, H. (1978). Morphogenesis in Thin Cell Layers: Concept, Methodology and Results. In T. A. Thorpe (ed.). Frontiers of Plant Tissue Culture. International Association for Plant Cell Culture.

Truscott, F.H. (1966). Some aspect of morphogenesis *in Cuscuta gronovii*. American Journal of Botany, 53, 739-750.

Tulecke, W. & McGranahan. (1985). Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regial*. Plant Science, 40, 57-63.

Vacin, E. & Went, F.W. (1949). Some pH changes in nutrient solutions. Botanical Gazette, 110, 605-613.

Verbeke, J.A. & Walker, D.B. (1986). Morphogenic factors controlling differentiation and dedifferentiation of epidermal cells in the gynoecium of *Catharanthus roseus*. II. Diffusible morphogens. Planta, 168, 43-49.

Vickery, J.W. (1933). Vegetative reproduction in *Drosera peltata* and *D. auriculata*. Proceedings of the Linnaen Society New South Wales, 58, 245-269.

Vidaver, W. (1977). Light and seed germination. In A.A. Khan (ed.). The Physiology and Biochemistry of Seed Dormancy and Germination. Amsterdam, The Nethertlands. North Holland Publishing Company.

Wallace, H.A.H. (1960). Factors affecting subsequent germination of cereal seeds sown in soils of subgermination moisture content. Canadian Journal of Botany, 38, 287-306.

Walker, D.B. (1975). Postgenital carpel fusion in *Catharanthus roseus* (Apocynaceae). I. Light and scanning electron microscopic study of gynoecial ontogeny. American Journal of Botany, 62, 457-467.

Walker, D.B. (1978). Morphogenic factors controlling differentiation and dedifferentiation of epidermal cells in the gynoecium of *Catharanthus roseus*. I. The role of pressure and cell confinement. Planta, 142, 181-186.

Wardlaw, C.W. (1968). Morphogenesis in Plants: A Contemporary Study. London, England. Methuen & Co., Ltd.

Weber, H., Heim, U., Golombek, S., Borisjuk, L., & Wobus, U. (1998). Assimilate uptake and the regulation of seed development. Seed Science Research, 8, 331-345.

Wetherell, D.F. & Halperin, W. (1963). Embryos derived from the callus tissue cultures of the wild carrot. Nature, 200, 336-1337.

White, J.F. Jr., Anthony, G.E., & Chandler, K.F. (1993). Endophyte-host associations in grasses. XVIII. Moisture relations and insect herbivory of the emergent stromal leaf of *Epichloe*. Mycologia, 85, 195-202.

White Jr., J.F. & Chambless, D.A. (1991). Endophyte-host associations in forage grasses. XV. Clustering of stomata-bearing individuals of *Agrostis hiemalis* infected by *Epichloe typhnia*. American Journal of Botany, 78, 527-533.

White, J.F. Jr. & Cole, G.T. (1986). Endophyte-host associations in forage grasses. IV. The endophyte of *Festuca versuta*. Mycologia, 78, 02-107.

White, J.F. Jr., Morrow, A.C., Morgan-Jones, G., & Chambless, D.A. (1991). Endophyte-host associations in forage grasses. XIV. Primary stomata formations and seed transmission in *Epichloe typhina*: developmental and regulatory aspects. Mycologia, 83, 72-81.

Withner, C.L. (1964). Notes on the cultivation of insectivorous plants *in vitro*. Bulletin of the Torrey Botanical Club, 91, 412-413.

Zenk, M.H., Furbringer, M., & Steglich, W. (1969). Occurrence and distribution of 7-methyljuglone and plumbagin in the *Droseraeceae*. Phytochemistry, 8, 2199-2200.

Ziv, M (1979) Transplanting *Gladiolus* plants propagated *in vitro*. Scientia Horticulturae, 11, 257- 260.